A STUDY OF VITAMIN SYNTHESIS BY YEAST

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DISSERTATION

Presented to the Faculty of the Graduate School of

The University of Texas in Partial Fulfillment

of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

By

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Austin, Texas

June, 1945

PREFACE

This study was suggested by, and carried out under the supervision of, Dr. Roger J. Williams. The work was expedited by the assistance of certain members of the staff of the Biochemical Institute in making vitamin assays. Funds supporting this work were supplied by Standard Brands, Inc. of New York for the sessions 1942-1943 and 1943-1944.

The author wishes to express her sincere appreciation to all of those who have made possible the undertaking and completion of this study.

May, 1945

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PART I

EFFECTS OF CERTAIN LIMITING CONDITIONS ON THE SYNTHESIS OF B VITAMINS BY YEAST

INTRODUCTION

The function of the B vitamins in metabolism is a challenging problem and to a large extent an unsolved one. The fact that these substances are present in all tissues and cells which have been examined lends support to the hypothesis that they are involved in the fundamental processes of life, though little is known concerning their specific functions. The relation of riboflavin, thiamine, and niacin to certain enzymic processes has been established definitely, but it remains to be seen whether or not the other B vitamins are similarly allied to enzyme systems and if the three nutrilites named have functions in addition to those which have been elucidated.

In microorganisms which possess the ability to synthesize all or most of the B vitamins and to retain relatively large amounts of them within their cells, it seems reasonable to suppose that fabrication of the vitamins is not merely an expression of functionless creative ability but rather is the response of the organisms to a requirement for those substances. If the medium used for culturing such organisms is modified, either by qualitative alterations or by the addition of certain metabolic inhibitors, it is likely that there will be a reflection of such modifications in the synthetic activities of the cells; and any correlation between changes in extent of synthesis of any two or several vitamins might serve as indirect evidence as to how these vitamins function.

In this investigation the effects of certain qualitative modifications in the culture medium on the total synthesis of B vitamins by a microorganism have been studied. The vitamin content of cells produced in qualitatively altered media has also been determined. Further, changes in

the vitamin content of cells cultured in the presence of various inhibitors have been measured, and an attempt has been made to interpret, or at least to speculate on, the significance of the observed variations in content. It is recognized that changes in total synthesis of the respective vitamins might be a preferred index, but by using changes in the cell content instead, the problem of removing the inhibitor from the culture prior to assay for the vitamins is avoided.

The organism chosen for this study was a strain of Saccharomyces cerevisiae which had been isolated from a cake of Fleischmann's yeast,[1] and which has been carried in pure culture in the laboratory of the Biochemical Institute at the University of Texas. This strain, designated as F.B. yeast, has been shown to have considerable synthetic ability. Williams, Eakin, and Snell[2] established the fact that an exogenous supply of thiamine, pyridoxine, and inositol is not required by this yeast and made the tentative assumption that synthesis of these nutrilites by the organism is the basis for their dispensability in the culture medium. These investigators found further that on prolonged incubation in a vitamin-free medium the yeast was able to grow very slowly, while in a medium containing biotin as the only added vitamin the growth observed was very nearly as extensive as that obtained in a medium containing addenda of thiamine, pyridoxine, inositol, pantothenic acid (or β-alanine), and biotin. Pantothenic acid, or its physiological equivalent, β-alanine, proved to be essential for immediate growth of F.B. yeast under the conditions employed in their study. Leonian and Lilly,[3] who

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[1] R. J. Williams, R. E. Eakin, and E. E. Snell, J. Am. Chem. Soc., 62, 1204 (1940).

[2] Ibid.

[3] L. H. Leonian and V. G. Lilly, Am. J. Bot., 29, 459 (1942).

also used a strain of yeast isolated from a Fleischmann's cake, reported that this yeast was unable to grow in a medium which contained only biotin or only pantothenic acid, while thiamine, pyridoxine, and inositol could be omitted with little or no effect on the extent of growth. In a later study of this same yeast, however, an increased "autotrophic" character was induced by successively subculturing the yeast in a medium from which first pantothenic acid, and then pantothenic acid and biotin were omitted.[4] The variant produced grew well in a medium free from thiamine, pyridoxine, pantothenic acid, and inositol, but in the absence of biotin, whether or not other vitamins were present, its growth was reduced.

It seemed desirable to repeat in part this earlier work and to extend it beyond a mere growth study by determining not only the extent of growth but also the amounts of the B vitamins synthesized by the yeast under various conditions.

The extent of growth of F.B. yeast during a period of successive transfer was determined in synthetic media modified by (1) substitution of other carbohydrates for sucrose and/or (2) omission of various vitamins. Casein hydrolysate was added to several of the media in which growth was scant in order to determine whether or not its presence would increase the amount of growth in the same manner that it does in a medium which supports relatively heavy growth of yeast.[5] Urea was also added to several of the media to serve as an additional source of nitrogen.

The extent of B vitamin synthesis in most of the qualitatively modified media was determined by assaying the acclimatized yeasts together

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[4] Ibid., J. Bact., 45, 329 (1943).

[5] H. K. Mitchell and R. J. Williams, Biochem. J., 34, 1532 (1940).

with the respective media in which they had grown. Only one of the qualitatively modified media employed in the growth study, a sucrose medium from which all of the B vitamins except biotin were omitted, was found to support sufficiently heavy growth to warrant separation of the yeast from the culture medium so that the cell content of the various vitamins could be determined. The vitamin content of yeast grown in this medium was determined so that respective values could be compared with those of yeast grown in a medium supplemented with certain members of the B group.

Bakers' yeast can be trained to utilize galactose after a brief period of acclimatization in a medium which contains galactose,[6] and the galac yeast thus derived from F.B. yeast was assayed for the B vitamins in order to determine what effect substitution of this carbohydrate for sucrose has on the vitamin content of yeast.

In studying the effects of various inhibitors on the vitamin content of yeast, reagents were chosen which, for the most part, have been shown to suppress fermentation, respiration, or growth of microorganisms. In some cases they are known to act on certain enzymic transformations, but it is highly probable that in no case are all of the sites of inhibition known.

The inhibitors were used in such concentration that they would, when added to a synthetic medium which supports heavy growth under control conditions, restrict the growth obtained in 24 hours but allow heavy growth in 72 hours, so that the yeast crops would be of sufficient size to permit harvesting and assay for the vitamins. The following inhibitors were used at the indicated concentrations: potassium cyanide (0.001 M), hydroxylamine (0.001 M), sodium sulfite (0.001 M), semicarbazide (0.01 M), 4,4'-diamidino-1,3-diphenoxypropane (0.001 M), sulfaguanidine (0.005 M), ethyl urethane

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[6] G. W. Kirby and L. Atkin, J. Biol. Chem., 116, 511 (1936).

(0.2 M), chloral hydrate (0.01 M), sodium fluoride (0.001 M), camphor (0.001 M), and camphor (saturated). Two levels of camphor were used because it was observed in preliminary studies that culture in the saturated medium produced involutional cell forms, but that the 0.001 M concentration had no such effect.

MODE OF ACTION OF THE INHIBITORS EMPLOYED

Four of the inhibitors used, viz., potassium cyanide, hydroxylamine, sodium sulfite, and semicarbazide, since they are reagents for the carbonyl group, might be expected to possess some degree of similarity in effect. In addition to their common ability to react with intermediary metabolites which contain a carbonyl group, these keto-fixatives might be expected to have certain individual and specific effects as enzyme inhibitors and as a result to cause differences in the vitamin content of cells cultured in their presence.

The following effects have been reported for the various inhibitors employed in this study:

Potassium Cyanide--In general, oxidases are inhibited by cyanide[7] while dehydrogenases are unaffected.[8, 9, 10, 11] The respiration of bakers' yeast is almost entirely inhibited by cyanide,[12, 13] and this effect has been attributed to the poisoning of cytochrome oxidase.[14, 15] Winzler,[16] however, recently reported that the effect of cyanide on yeast respiration

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[7] D. Keilin, Proc. Roy. Soc. London, Series B, 104, 206 (1928).

[8] L. F. Leloir and M. Dixon, Enzymologia, 2, 81 (1937).

[9] M. Stephenson, Biochem. J., 22, 605 (1928).

[10] E. F. Gale and M. Stephenson, ibid., 33, 1245 (1939).

[11] J. R. Hawthorne and D. C. Harrison, ibid., 1573 (1939).

[12] E. Negelein, Biochem. Z., 165, 203 (1925).

[13] M. Dixon and K. A. C. Elliot, Biochem. J., 23, 812 (1929).

[14] D. Keilin, op. cit.

[15] Ibid., Proc. Roy. Soc. London, Series B, 98 312 (1925).

[16] R. J. Winzler, J. Cellular Comp. Physiol., 21, 229 (1943).

depends not only on the poisoning of cytochrome oxidase but also on the inhibition of the enzyme system which normally is the pace-setting reaction in oxygen consumption. Potter[17] found the enzymatic reduction of cytochrome c to be blocked by cyanide, and his results indicated that cytochrome c was the locus of the action; the enzyme responsible for the change was unaffected. Winzler[18] has suggested that this may be the cyanide sensitive pace-making reaction in yeast respiration.

Burkholder[19] found that the addition of small amounts of cyanide to growing cultures of yeast inhibited growth but stimulated riboflavin synthesis by the cells. Pett[20] reported that the flavin content of yeast was increased by culture in a cyanide medium; the Q_{O_2} of such a yeast after removal from the cyanide medium was approximately one-fourth that of the control yeast and was scarcely affected by cyanide, while the Q_{CO_2} was usually higher than that of the control and was not increased by determination in nitrogen.

According to Massart and Dufait,[21] alcoholic fermentation was suppressed by cyanide, and the inhibition could be localized in the ester exchange reactions which are catalyzed by the phosphopyruvic acid-adenylic acid phosphopherase and the phosphoadenylic acid-glucose phosphopherase.

Cyanide inhibits catalase,[22] the enzyme in yeast responsible for the decomposition of hydrogen peroxide.

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[17] V. R. Potter, J. Biol. Chem., 137, 13 (1941).

[18] R. J. Winzler, op. cit.

[19] P. R. Burkholder, Proc. Nat. Acad. Sci., 29, 166 (1943).

[20] L. B. Pett, Biochem. J., 30, 1438 (1936).

[21] L. Massart and R. Dufait, Z. physiol. Chem., 272, 157 (1942).

[22] D. Keilin, Proc. Roy. Soc. London, Series B, 121, 173 (1936).

Hydroxylamine--Hydroxylamine inhibits the anaerobic fermentation of Fleischmann's bakers' yeast.[23] It is an inhibitor of catalase,[24] combining with the hematin of reduced catalase.[25]

Sodium Sulfite--The addition of sodium sulfite to a fermenting mixture of yeast and sugar causes a reduced yield of alcohol and carbon dioxide with considerable amounts of glycerol and acetaldehyde (in equivalent quantities) being formed; the acetaldehyde is present as the bisulfite compound.[26, 27, 28]

Semicarbazide--Although no reports on the action of this compound on living yeast or on yeast processes have been found, semicarbazide was included in the group of inhibitors employed because it is a reagent for the carbonyl group.

4,4'-Diamidino-1,3-diphenoxypropane (Propamidine)--No studies have been reported in which propamidine has been used as a yeast inhibitor. It inhibited the growth of Lactobacillus casei and Streptococcus lactis, the inhibition being prevented by the simultaneous addition of certain polyamines.[29] The ability of Escherichia coli to oxidize certain amino acids was inhibited by propamidine, but the inhibitor had relatively no

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[23] C. V. Smythe, Enzymologia, 6, 9 (1939).

[24] D. Keilin, Proc. Roy. Soc. London, Series B, 121, 173 (1936).

[25] M. G. Sevag, M. Shelburne, and M. Ibsen, J. Biol. Chem., 144, 711 (1942).

[26] C. Neuberg and E. Reinfurth, Biochem. Z., 89, 365 (1918).

[27] Ibid., 92, 234 (1918).

[28] W. Connstein and K. Lüdecke, Ber., 52 B, 1385 (1919).

[29] E. E. Snell, J. Biol. Chem., 152, 475 (1944).

effect on the ability of this organism to oxidize glucose, pyruvic acid, or succinic acid.[30]

Sulfaguanidine--Landy and Dicken[31] found that sulfaguanidine completely inhibited the growth of a strain of Saccharomyces cerevisiae in a 16 hour growth period and that the inhibition was reversed by the addition of p-aminobenzoic acid to the culture.

Ethyl Urethane--Urethane has some depressive effect on the total oxygen uptake by yeast cells, and its effect is principally inhibition of the reducing systems of the cell, i.e., dehydrogenases, not oxidases.[32,33,34] Using urethane as an inhibitor, Fisher and Stern[35] obtained evidence for the existence of two parallel respiratory systems in yeast which together are responsible for the total respiration of the organism. One of the systems appears to be more sensitive to urethane than the other and is almost completely inhibited when the total respiration is 30 per cent inhibited. Results indicated that the more sensitive system is the one more closely correlated with the metabolic changes which are connected with cell division.

Chloral Hydrate--Chloral hydrate, when added to yeast juice, was observed to have a slightly stimulative effect on fermentation.[36] No report of its action on living yeast has been found.

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[30] F. Bernheim, J. Pharmacol., 80, 199 (1944); Chem. Abstr., 38, 2064 (1944).

[31] M. Landy and D. M. Dicken, Nature, 149, 244 (1942).

[32] D. Keilin, Proc. Roy. Soc. London, Series B, 98, 312 (1925).

[33] Ibid., 104, 206 (1928).

[34] Ibid., 106, 418 (1930).

[35] K. C. Fisher and J. R. Stern, J. Cellular Comp. Physiol., 19, 109 (1941).

[36] F. Duchacek, Biochem. Z., 18, 211 (1909).

Sodium Fluoride—This reagent caused a strong inhibition of the hydrolytic action of phosphatases present in both muscle and yeast preparations.[37, 38] It prevented both the fermentation and phosphorylation of glucose by yeast maceration juice, but with glycogen the fermenting action of the juice was reduced 70 per cent by fluoride, while its phosphorylating action was lowered only 23 per cent.[39, 40] According to Cori, the phosphorylation of glycogen to yield glucose-1-phosphate is not a fluoride sensitive reaction.[41] But in living cells both formation[42, 43] and breakdown[44] of glycogen are reported to be inhibited by fluoride.

The fluoride inhibition of enolase reported by Meyerhof[45, 46] has been confirmed using a crystalline preparation of the enzyme.[47] This is the enzyme which catalyzes the conversion of 2-phosphoglyceric acid to phosphopyruvic acid in the chain of fermentation reactions, and its fluoride sensitivity is undoubtedly responsible in part for the inhibition of fermentation observed in the presence of fluoride. There is considerable evidence

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[37] F. Lipmann, Biochem. Z., 196, 3 (1928).

[38] L. Massart and R. Dufait, Z. physiol. Chem., 272, 157 (1942).

[39] F. Lipmann, op. cit.

[40] O. Meyerhof, Biochem. Z., 183, 176 (1927).

[41] G. T. Cori, S. P. Colowick, and C. F. Cori, J. Biol. Chem., 127, 77 (1939).

[42] E. Wertheimer, Protoplasma, 21, 522 (1934).

[43] J. Runnström, R. Gurney, and E. Sperber, Enzymologia, 10, 1 (1942).

[44] Ibid.

[45] K. Lohmann and O. Meyerhof, Biochem. Z., 273, 60 (1934).

[46] O. Meyerhof, Ergebnisse Physiol., 39, 10 (1937).

[47] O. Warburg and W. Christian, Biochem. Z., 310, 384 (1941).

that enolase is identical with the enzymes serine deaminase and cysteine desulfurase;[48] part of the evidence for the identity of the three preparations is fluoride sensitivity.

Both the oxidation and decarboxylation of pyruvic acid by bakers' yeast were blocked by fluoride,[49] and a pure preparation of carboxylase has been shown to be inhibited by this poison.[50] Carboxylase is not as sensitive to fluoride as is enolase, however.

Fluoride has been found to prevent the absorption of thiamine by bakers' yeast,[51] to cause a reduction in cozymase content, and to destroy the ability of the cells to synthesize cozymase from glucose, adenine, and nicotinamide.[52]

Camphor--This reagent has been used to induce polyploidy in yeast.[53,54]

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[48] F. Binkley, J. Biol. Chem., 150, 261 (1943).

[49] J. Runnström and K. Brandt, Arkiv Kemi, Mineral. Geol., 15 A, 29 (1941); Chem. Abstr., 36, 5205 (1942).

[50] O. Warburg and W. Christian, op. cit.

[51] E. Sperber, Biochem. Z., 313, 62 (1942).

[52] A. Lennerstrand, Arkiv Kemi, Mineral. Geol., 14 A, 1 (1941); Chem. Abstr., 36, 793 (1942).

[53] R. Bauch, Wochschr. Brau., 59, 1 (1941); Chem. Abstr., 36 6573 (1942).

[54] A. Levan and C. G. Sandwall, Hereditas, 29, 164 (1943); Chem. Abstr., 37, 6692 (1943).

EXPERIMENTAL

Culture Conditions Employed

Medium--The basal medium used in this study is that of Williams;[55] its composition is as follows:

Sucrose	20 gm.
$(NH_4)_2SO_4$	3 gm.
KH_2PO_4	2 gm.
l-Aspartic acid	0.1 gm.
$CaCl_2$	0.25 gm.
$MgSO_4 \cdot 7H_2O$	0.25 gm.
H_3BO_3, $ZnSO_4$, $MnCl_2$, $TlCl_3$	1 mg. each
$CuSO_4 \cdot 5H_2O$	0.1 mg.
KI	0.1 mg.
Distilled H_2O to make	1 liter

The medium referred to in the text as the supplemented medium contained the following addenda of vitamins per liter:

Inositol	5 mg.
β-Alanine	0.5 mg.
Thiamine hydrochloride	20 γ
Pyridoxine hydrochloride	20 γ
Biotin (crystalline)	1 γ

Incubation Temperature--The incubation temperature used throughout this study was 30° C.

Measurement of Growth--The amount of growth at the end of the incubation period was determined by measurement with a thermocouple turbidimeter, or by harvesting, drying, and weighing the yeast crops.

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[55] R. J. Williams, Univ. Texas Pub., 4237, 7 (1942).

Methods Used for Vitamin Assays

In making the vitamin assays, microbiological methods were used exclusively. Inositol, biotin, riboflavin, pantothenic acid, niacin, folic acid, and thiamine assays were made using the methods summarized by Williams.[56] Thiamine determinations on most of the samples were also made using the microbiological method of Niven and Smiley.[57] p-Aminobenzoic acid values were obtained using the test developed by Lewis,[58] and vitamin B_6 assays were made according to the method of Atkin et al.[59]

Determination of Extent of Growth of Yeast Cultured in Qualitatively Modified Media

In order to study the effects of certain qualitative modifications in the nutrient medium on the growth of F.B. yeast, the following media were employed:

(1). Basal medium

(1a). Basal medium + 200 mg. per liter of casein hydrolysate (twice treated with 10 per cent Norit)

(1b). Basal medium + 40 mg. per liter of urea

(2). Basal medium + vitamin supplement with biotin omitted

(2a). Basal medium + vitamin supplement with biotin omitted + 200 mg. per liter of casein hydrolysate (twice treated with 10 per cent Norit)

(2b). Basal medium + vitamin supplement with biotin omitted + 40 mg. per liter of urea

(3). Basal medium + 1 γ per liter of biotin ("biotin medium")

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[56] Ibid.

[57] C. F. Niven and K. L. Smiley, J. Biol. Chem., 150, 1 (1943).

[58] J. C. Lewis, ibid., 146, 441 (1942).

[59] L. Atkin, A. S. Schultz, W. L. Williams, and C. N. Frey, Ind. Eng. Chem., Anal. Ed., 15, 141 (1943).

(4). Basal medium with sucrose replaced by maltose (20 gm. per liter)

(5). Basal medium with sucrose replaced by xylose (20 gm. per liter)

(6). Supplemented medium with sucrose replaced by xylose (20 gm. per liter)

(6a). Supplemented medium with sucrose replaced by xylose (20 gm. per liter) + 200 mg. per liter of casein hydrolysate (twice treated with 10 per cent Norit)

Duplicate sterile portions (50 ml. in 125 ml. Erlenmeyer flasks) of media (1), (2), (3), (4), (5), and (6) were inoculated with 0.3 mg. of moist F.B. yeast from a 24 hour inoculum grown in the supplemented medium. These were then incubated for 124 hours. The extent of growth was determined at the end of this period, and the yeast was then successively subcultured over an extended period of time in the respective media, growth measurements being made at the end of each incubation period.

On the eighth transfer, yeast cells cultured in media (1), (2), and (6) were transferred into similar media (1a), (2a), and (6a), respectively, for a period of successive subculturing. On the eleventh transfer, media (1b) and (2b) were seeded with yeast cultured in media (1) and (2), respectively, and consecutive transfers were made in these media.

The yeast crops resulting from periodic transfer in these various media are tabulated in Table I together with the respective incubation intervals and inoculums used.

Determination of Extent of Vitamin Synthesis by Yeast Cultured in Qualitatively Modified Media

In the case of cultures grown in media (1), (1b), (2), (2b), (6), and (6a), the yeast cells together with the medium in which they had grown

TABLE I

Extent of Growth of F.B. Yeast Successively Subcultured in Various Qualitatively Modified Media

Culture sequence	Seeding (moist yeast)	Incubation period	Extent of growth* in various media											
			Medium (1)	Medium (1a)	Medium (1b)	Medium (2)	Medium (2a)	Medium (2b)	Medium (3)	Medium (4)	Medium (5)	Medium (6)	Medium (6a)	Supplemented medium (control)
	mg. per culture	hrs.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
1	0.3	124	7			40			58	19	12	20		195
2	0.3	136	1			1.2			105	4.5	0.3	17		218
3	0.3	120	1.8			1.8			113	5.3		19		200
4	0.3	72	4.8			25			170	4.5		13		180
5	0.2	72	13			29			205	11		12		190
6	0.2	72	11			11			170	12		12		180
7	0.2	90	15			11			220	14		17		
8	0.2	72	9.5			5				11		11		
9	0.2	72	9.3	8		6	5.5					11	12	
10	0.2	72	11	13		4	4					11	13	
11	0.2	168	30	21		7	11					17	24	
12	0.2	168	25	22	35	10	8.7	13				20	26	
13	0.2	192	28		25	16		23				14	21	
14	0.2	192	19		20	10		11				22		
15	0.2	168	24			14						8		

*The extent of growth is expressed as mg. of moist yeast per 50 ml. of culture medium.

for 8 days (cultures No. 13, Table I) were assayed for the B vitamins. For those cultures containing added amounts of several of the vitamins, assay values were corrected for the amount of each vitamin added.

Samples for assay were prepared in the following manner. The medium containing the yeast crop was steamed for 30 minutes, cooled, brought to pH 4.5 by the addition of NaOH, and, after the addition of 10 mg. per culture of a 1:1 mixture of clarase and papain, was incubated under benzene for 24 hours at 37° C. The mixture was then steamed for 30 minutes to inactivate the enzymes, and an aliquot was removed to be acid extracted. The remaining portion was filtered through kieselguhr, and after adjustment of the volume was assayed for pantothenic acid, niacin, riboflavin, folic acid, thiamine, and inositol.

The aliquot removed prior to filtration was made 2 N in HCl and autoclaved for 1 hour. After cooling, the pH was adjusted to 4.5 by the addition of NaOH, and the extract was filtered through kieselguhr. This extract was assayed for biotin, p-aminobenzoic acid, vitamin B_6, and inositol.

Results of the assays are presented in Table II; for comparative purposes, the vitamin content of yeast cells grown in the supplemented medium is included.

Determination of Vitamin Content of Yeast Cultured in Qualitatively Modified Media

After approximately a month's transfer in medium (3), a crop of the resulting "biotin yeast" was prepared in the following way. Each of two 2000 ml. Erlenmeyer flasks containing 500 ml. of "biotin medium" was seeded with 2 mg. of moist "biotin yeast" from a 72 hour inoculum and incubated

TABLE II

Amounts of B Vitamins Synthesized by F.B. Yeast in Various Qualitatively Modified Media Compared with the Vitamin Content of Cells Grown in the Control Medium

Material assayed	Yeast crop*	Thiamine†	Niacin	Pantothenic acid	Inositol (acid-extracted)	Inositol (enzyme-extracted)	Riboflavin	Folic acid‡	Vitamin B_6	p-Aminobenzoic acid	Biotin
Cells + culture medium from	mg. per liter	γ per liter	γ per liter	γ per liter	γ per liter	γ per liter	γ per liter	γ per liter	γ per liter	γ per liter	γ per liter
Medium (1)	560	40	520	160	5,100	100	60	25	240	42	0.071
Medium (1b)	490	40	480	120	3,900	0	80	27	230	49	0.088
Medium (2)	320	45	530	110	9,800	1,100	40	12	50	8.1	0.062
Medium (2b)	450	64	630	80	11,000	2,200	40	14	60	9.2	0.065
Medium (6)	280	58	270	60	1,300	2,400	20	3	Toxic	0.32	0
Medium (6a)	420	79	340	50	2,400	2,100	40	4	Toxic	0.83	0
Yeast separated from 1 liter of supplemented medium	3,800	100	600	46	1,400		37	24	19	24	0.7

*The amount of yeast produced is expressed on a moist basis.

†Thiamine values were determined by the yeast growth assay method.

‡Folic acid values represent micrograms of material of "potency 40,000".

for 72 hours. The yeast was separated from the culture medium by centrifugation, washed twice with 50 ml. portions of distilled water, and after the last centrifugation, transferred to a filter paper to drain free of excess water. It was then forced through a wire mesh, air dried for 48 hours, and placed in a desiccator over $CaCl_2$ for 48 hours.

The dried yeast was treated with enzymes according to the method of Cheldelin et al.,[60] and the resulting extract was assayed for pantothenic acid, niacin, riboflavin, folic acid, and thiamine. Preliminary studies were made to determine the most satisfactory extraction procedures for the remaining vitamins, and the following treatments were found to result in maximum liberation of the respective nutrilites from dried yeast. For biotin and inositol, the yeast was autoclaved for 1 hour in 20 volumes of 3 N HCl, and for p-aminobenzoic acid and vitamin B_6, it was autoclaved for 30 minutes with 20 volumes of 2 N H_2SO_4. Following acid hydrolysis, NaOH was added to each preparation to bring the pH to 4.5, and the extracts were then filtered through kieselguhr.

Galac yeast was produced by successive subculture in a medium containing galactose (25 gm. per liter) and Difco yeast extract (15 gm. per liter).[61] Three crops of the dried yeast from different stages of the subculturing were extracted in the same manner as that described for the "biotin yeast", and the resulting extracts were assayed. The crops selected for assay were those obtained on the second, sixth, and eighth transfers in the galactose medium, so that any trend in changing content of the respective vitamins might be discerned.

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[60] V. H. Cheldelin, M. A. Eppright, E. E. Snell, and B. M. Guirard, Univ. Texas Pub., 4237, 15 (1942).

[61] R. Johnston, Unpublished work.

Assay values for the "biotin yeast" and for crops Nos. 2, 6, and 8 of the galac yeast are shown in Table III.

Determination of Vitamin Content of Yeast Cultured in the Presence of Various Inhibitors

In order to determine what concentration of each inhibitor used would restrict growth in a 24 hour period but permit heavy growth in 72 hours, varying amounts of the respective inhibitors were added aseptically to duplicate 20 ml. portions of sterile supplemented medium distributed in 50 ml. Erlenmeyer flasks. The solutions were seeded with 0.2 mg. of moist yeast from a 24 hour inoculum, and after 72 hours were read turbidimetrically in order to determine how much growth had taken place. As a control, the extent of growth in similar portions of the supplemented medium was determined.

As a result of these preliminary studies, the following concentrations of the respective inhibitors seemed suitable: potassium cyanide (0.001 M), hydroxylamine (0.001 M), sodium sulfite (0.001 M), semicarbazide (0.01 M), propamidine (0.001 M), sulfaguanidine (0.005 M), ethyl urethane (0.2 M), chloral hydrate (0.01 M), sodium fluoride (0.001 M), camphor (0.001 M), and camphor (saturated). These concentrations were obtained by adding the calculated weight of inhibitor to the sterile medium just prior to inoculation with yeast.

A crop of yeast was grown in the presence of each inhibitor at the indicated concentration, using the same procedure as that described for preparing the "biotin yeast". The medium employed was the supplemented medium with added inhibitor, and the inoculum used was F.B. yeast from a

24 hour culture. The yeast from each culture was harvested, dried, and extracted following the procedure already described.

In the cyanide medium it was necessary to extend the incubation period since no discernible growth had occurred in 72 hours. Even after 9 days no growth was apparent in the flasks; therefore one flask was put on a shaker for 48 hours, at the end of which time an increase in turbidity was noted. Three days later the yeast was harvested from this medium. In the flask which was not shaken, an increase in turbidity was observed after 13 days, and the yeast was harvested after 16 days of incubation.

For control purposes, a crop of F.B. yeast was grown in the supplemented medium, harvested, and assayed using the procedures previously indicated.

Assay results together with crop yields are presented in Table III.

In order to simplify the comparative study of changes in content of one or several vitamins, a device has been adopted which was first employed by Wright et al.[62] The vitamin content of each yeast crop has been compared with that of the control yeast by expressing the respective assay values in terms of percentage of the corresponding values for the control. A series of "vitamin profiles" are shown in Figs. 1-16; "profiles" are included for the yeasts which were grown in the presence of inhibitors (Figs. 1-12), for the "biotin yeast" (Fig. 13), and for the galac yeasts (Figs. 14-16). The "profile" for the control yeast has been indicated in each graph by means of a horizontal line.

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[62] L. D. Wright, J. R. McMahan, V. H. Cheldelin, A. Taylor, E. E. Snell, and R. J. Williams, Univ. Texas Pub., 4137, 38 (1941).

TABLE III

Vitamin Content of Yeast Cultured in Various Kinds of Media

Yeast crop from	Dry weight of crop	Thiamine (Streptococcus salivarius test)	Thiamine (yeast growth test)	Niacin	Pantothenic acid	Inositol	Riboflavin	Folic acid*	Vitamin B_6	p-Aminobenzoic acid	Biotin
	gm. per liter	γ per gm.†	γ per gm.	γ per gm.	γ per gm.	γ per gm.	γ per gm.	γ per gm.	γ per gm.	γ per gm.	γ per gm.
Supplemented medium (control)	1.2	30	84	500	39	1,100	30	20	16	20	0.57
Cyanide medium (shaken culture)	1.2	14	62	400	81	980	38	4.0	16	17	0.58
Cyanide medium (unshaken culture)	1.2	17	72	360	100	810	45	4.3	11	36	1.4
Hydroxylamine medium	1.1	15	40	330	46	820	30	10	17	9.8	1.6
Sulfite medium	1.2	18	64	270	9.3	770	35	46	19	35	0.83
Semicarbazide medium	0.70	11	27	54	4.1	730	14	9.2	3.0	9.7	1.2
Propamidine medium	0.90	30	78	520	32	570	25	20	9.9	24	1.2
Sulfaguanidine medium	0.81	34	90	750	18	1,600	45	5.6	40	13	2.4
Urethane medium	0.94	28	100	520	4.8	980	25	23	5.9	20	0.69
Chloral hydrate medium	1.1	28	73	400	32	850	22	2.5	47	19	0.78
Fluoride medium	0.48	12	27	38	12	720	4.4	0.72	16	2.4	1.6
Camphor medium (0.001 M)	1.2	30	78	610	31	980	42	13	13	17	0.84
Camphor medium (saturated)	0.54	21	82	730	24	810	25	2.5	3.0	8.5	1.6
"Biotin medium"	1.2	27	100	760	31	770	31	23	31	41	0.59
Galactose medium (crop No. 2)		60	250	440	79	1,100	38	4.2	24	9.2	4.8
Galactose medium (crop No. 6)		17	65	540	61	1,600	38	3.1	28	34	0.56
Galactose medium (crop No. 8)		29	80	440	37	1,500	36	6.3	47	18	0.49

*Folic acid values represent micrograms of material of "potency 40,000".

†All vitamin contents are expressed as micrograms per gm. of dry yeast.

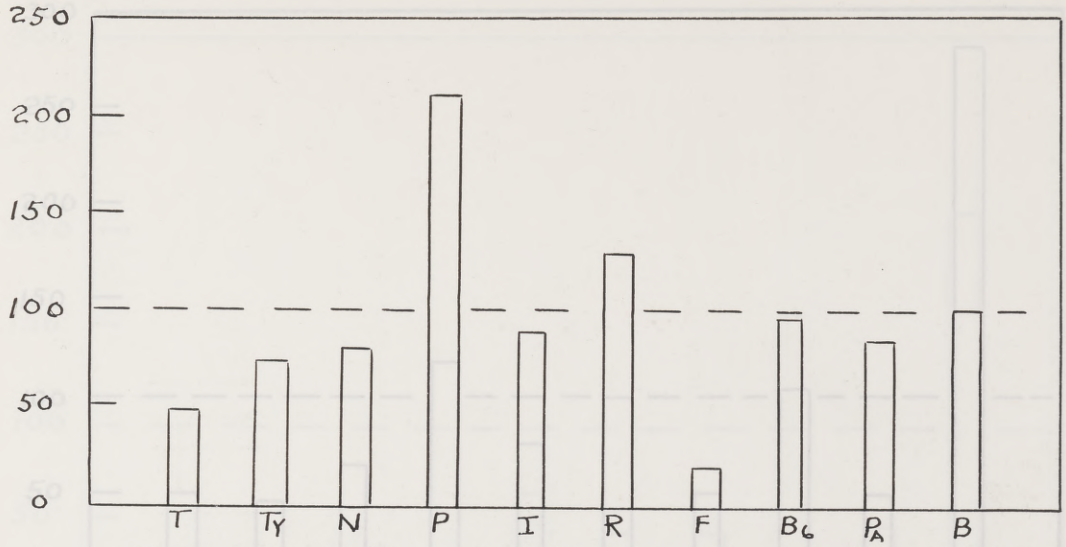

Fig. 1. "Vitamin profile" for yeast from the cyanide medium (shaken culture). For Figs. 1-16, abscissa symbols are defined as follows: T = thiamine (Streptococcus salivarius assay), T_Y = thiamine (yeast growth assay), N = niacin, P = pantothenic acid, I = inositol, R = riboflavin, F = folic acid, B_6 = vitamin B_6, P_A = p-aminobenzoic acid, and B = biotin; ordinate values represent 100 x (content of derived yeast ÷ content of control yeast).

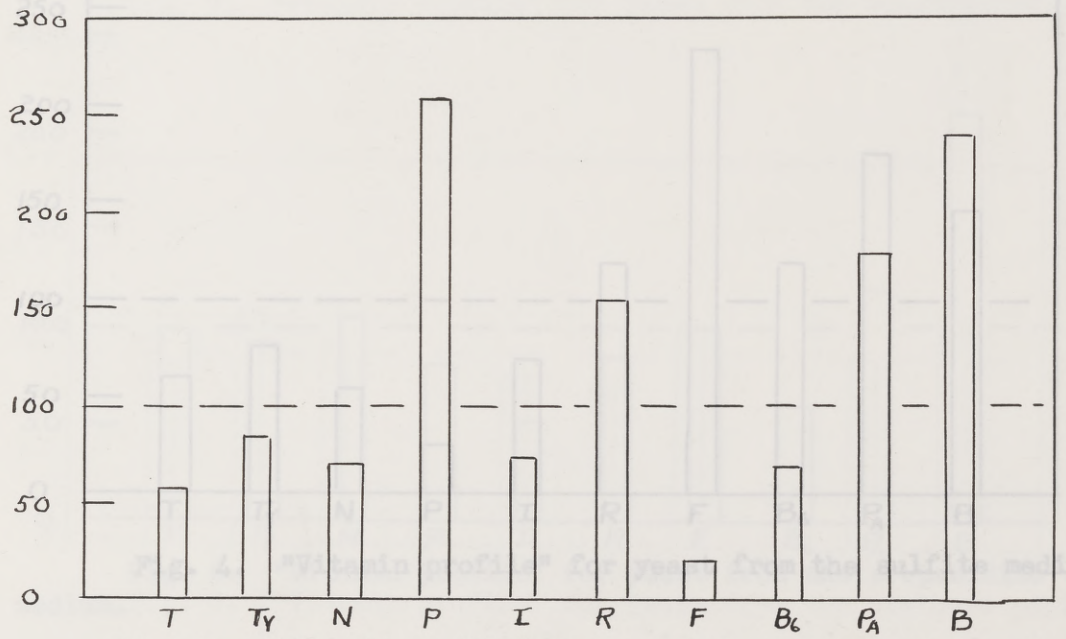

Fig. 2. "Vitamin profile" for yeast from the cyanide medium (unshaken culture).

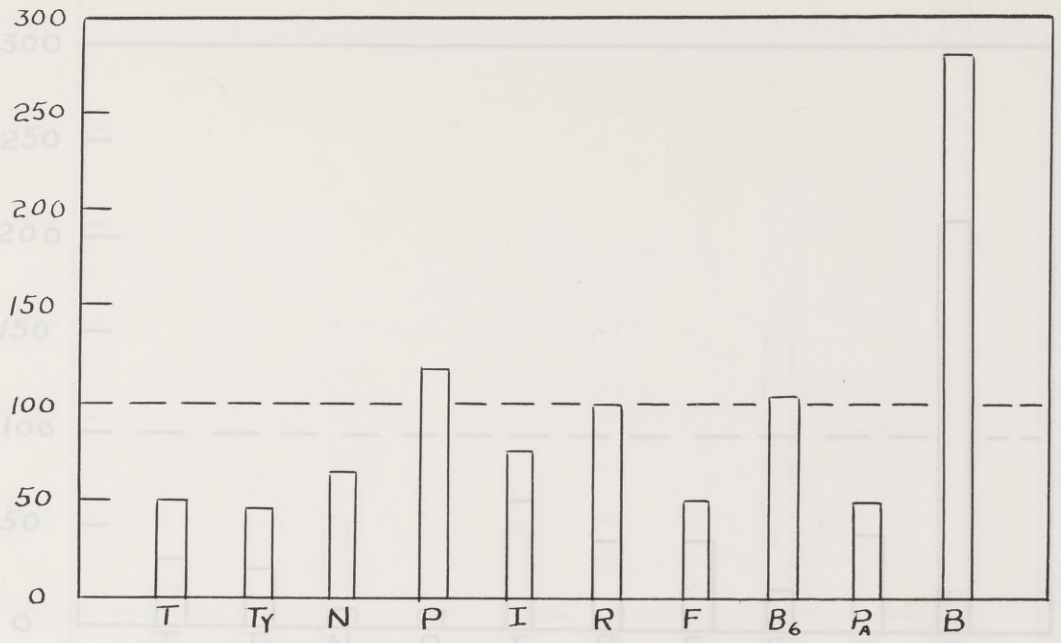

Fig. 3. "Vitamin profile" for yeast from the hydroxylamine medium.

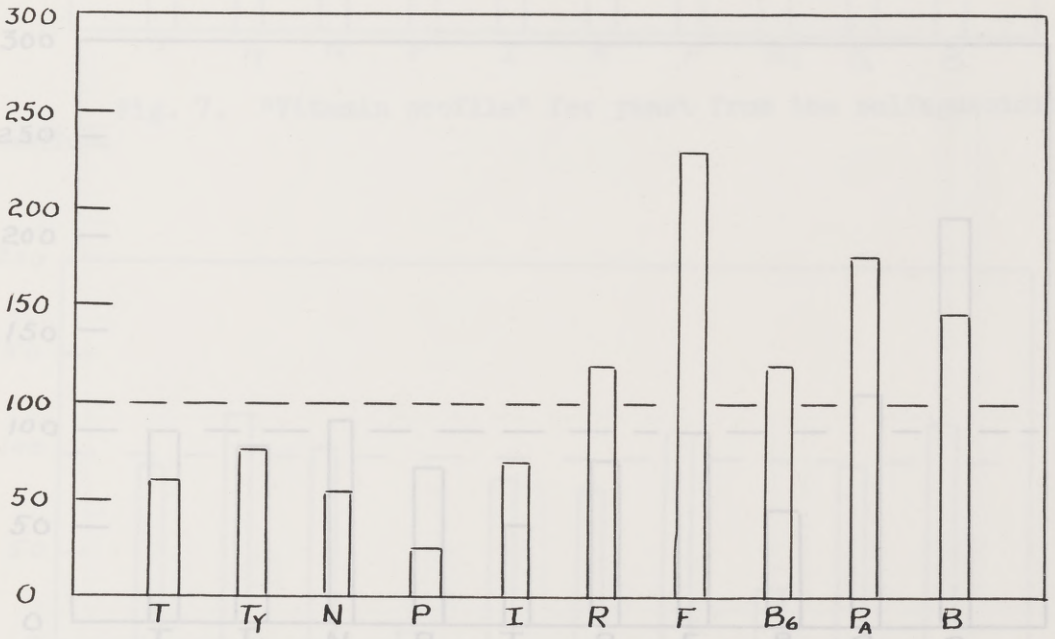

Fig. 4. "Vitamin profile" for yeast from the sulfite medium.

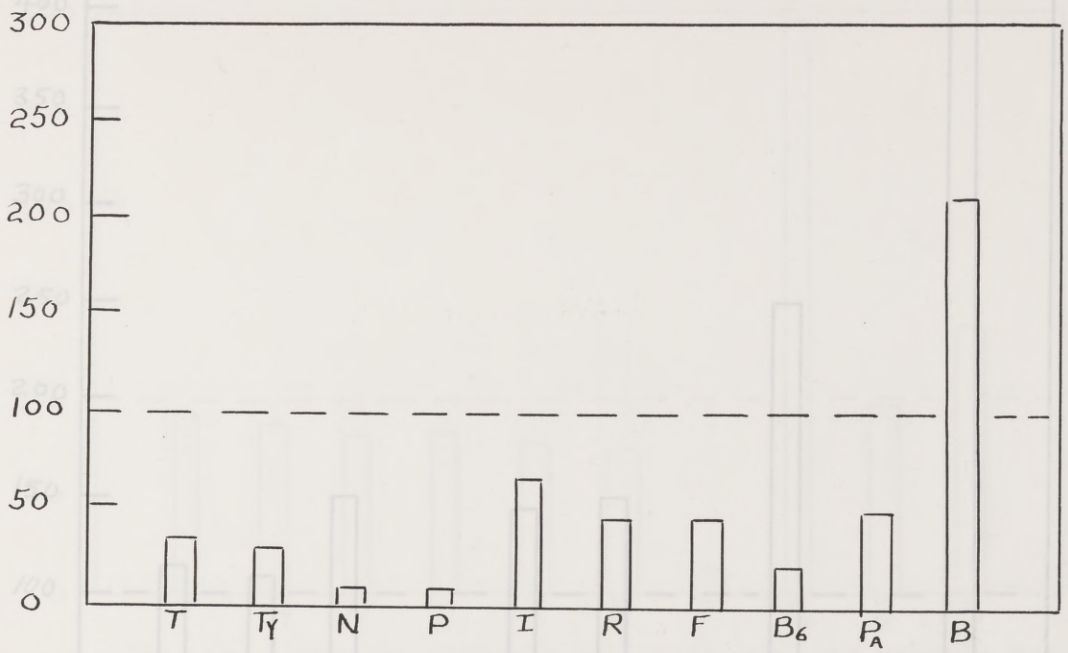

Fig. 5. "Vitamin profile" for yeast from the semicarbazide medium.

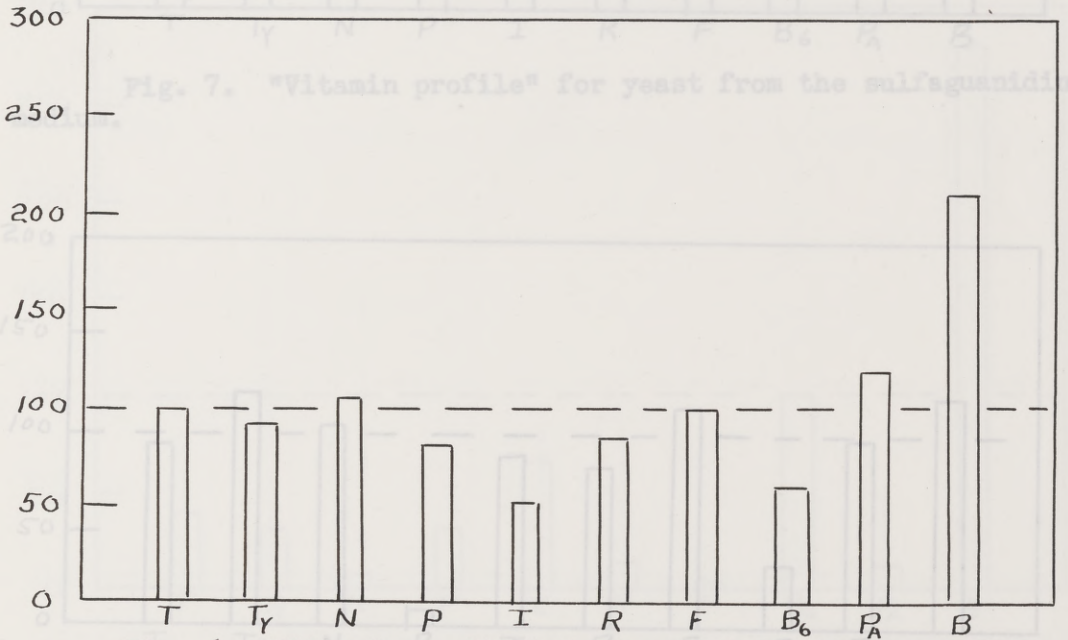

Fig. 6. "Vitamin profile" for yeast from the propamidine medium.

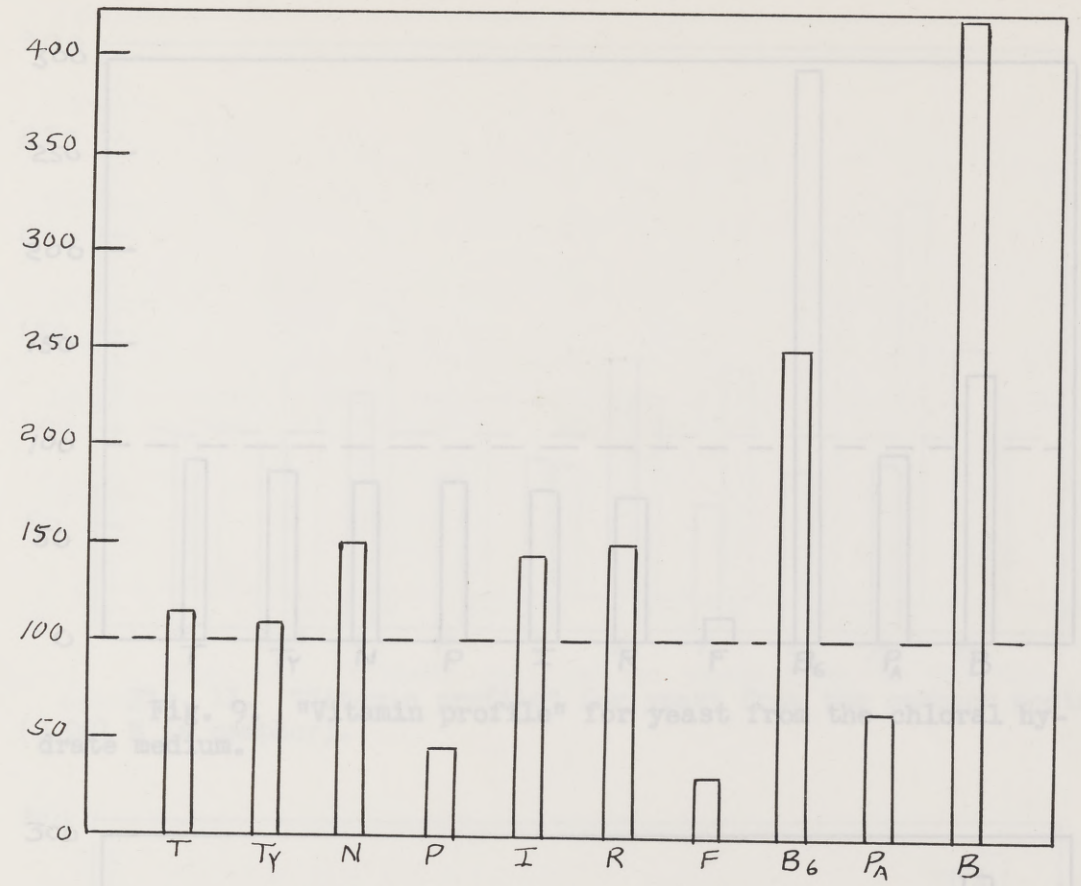

Fig. 7. "Vitamin profile" for yeast from the sulfaguanidine medium.

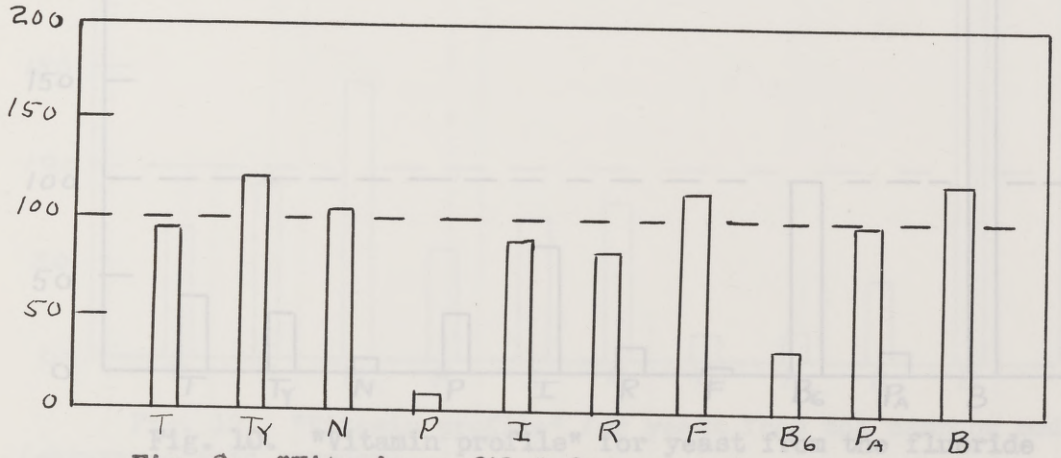

Fig. 8. "Vitamin profile" for yeast from the urethane medium.

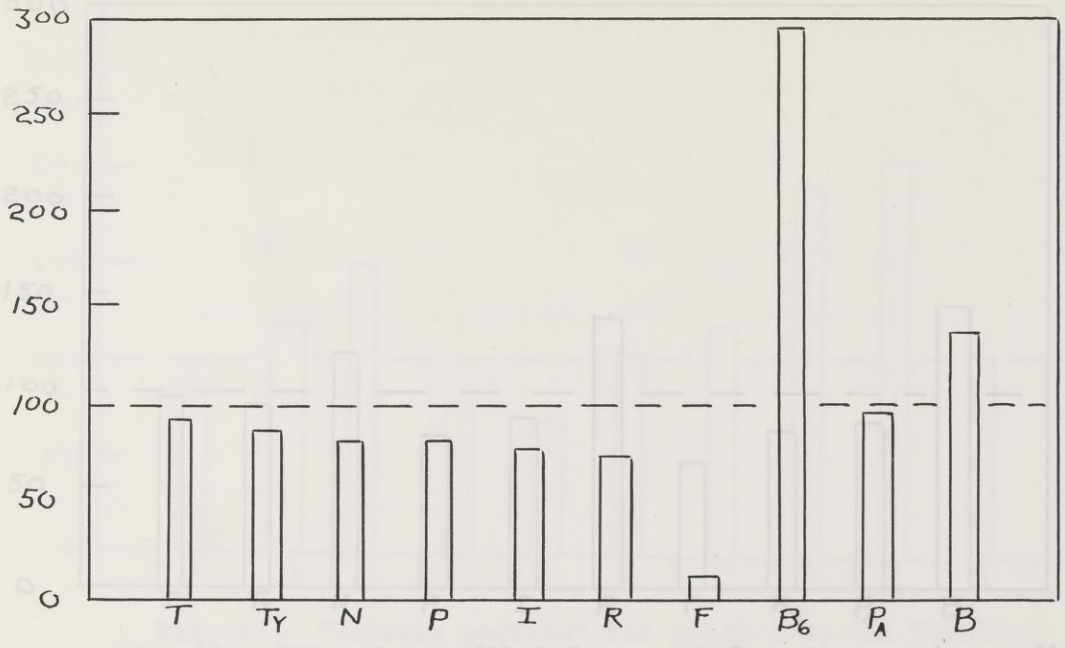

Fig. 9. "Vitamin profile" for yeast from the chloral hydrate medium.

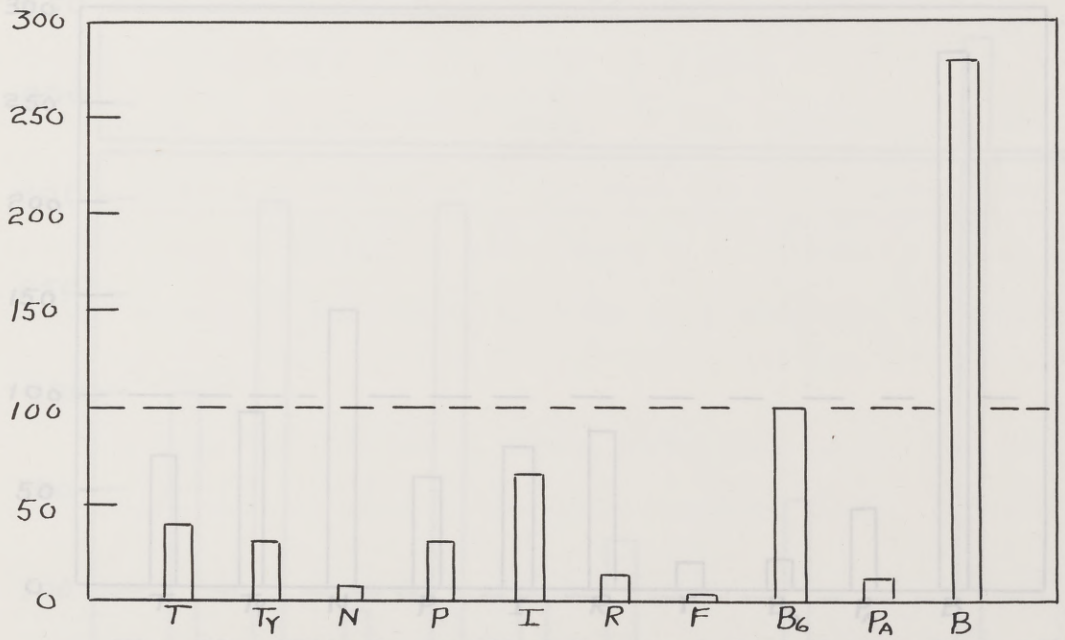

Fig. 10. "Vitamin profile" for yeast from the fluoride medium.

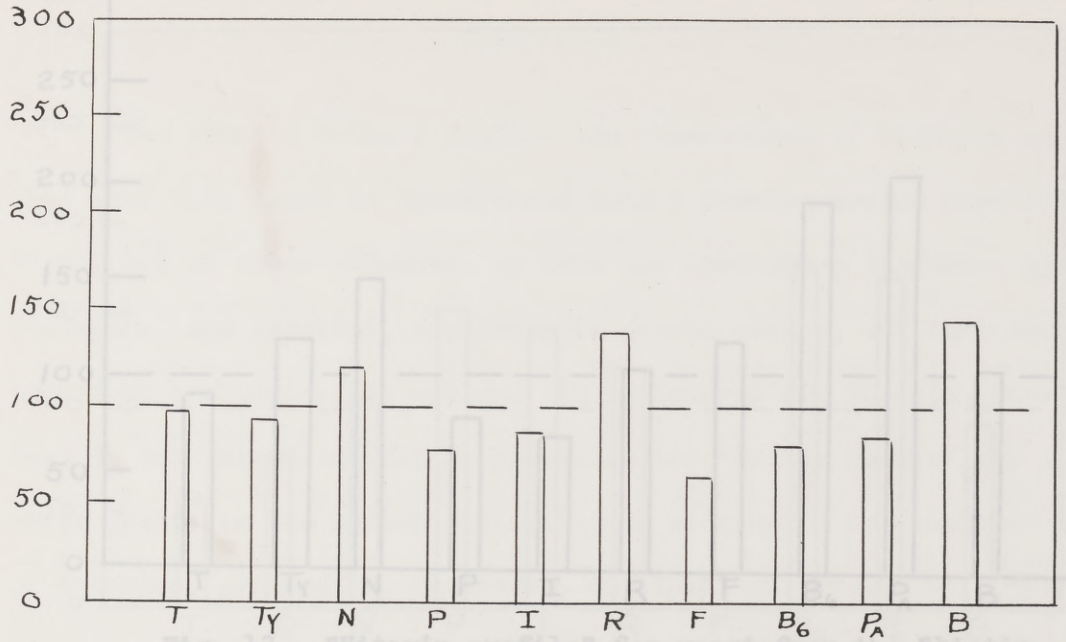

Fig. 11. "Vitamin profile" for yeast from the camphor medium (0.001 M in camphor).

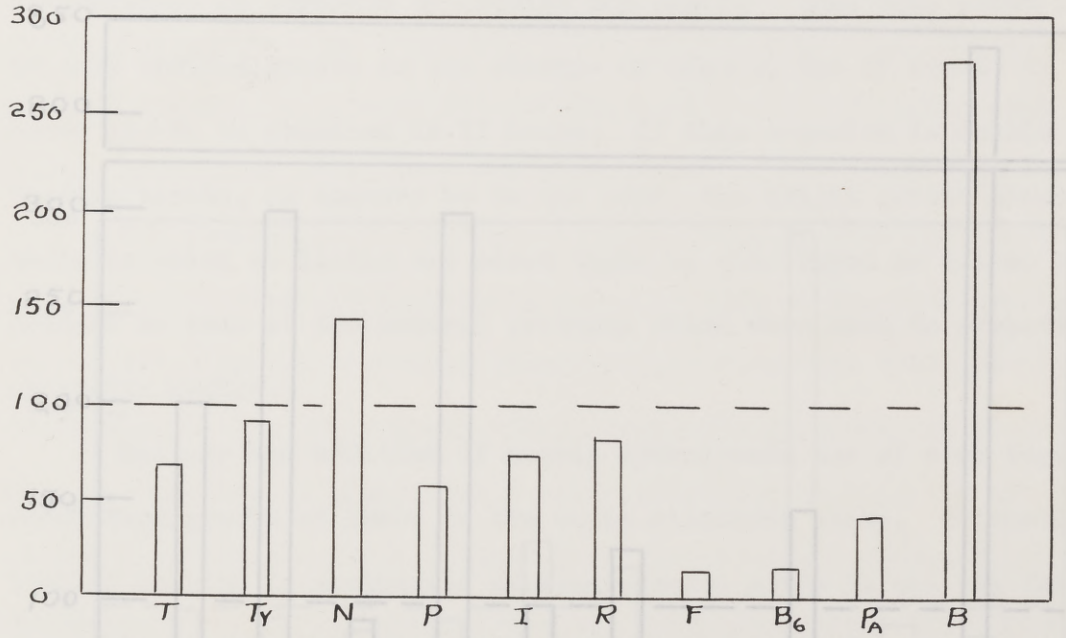

Fig. 12. "Vitamin profile" for yeast from the camphor medium (saturated with camphor).

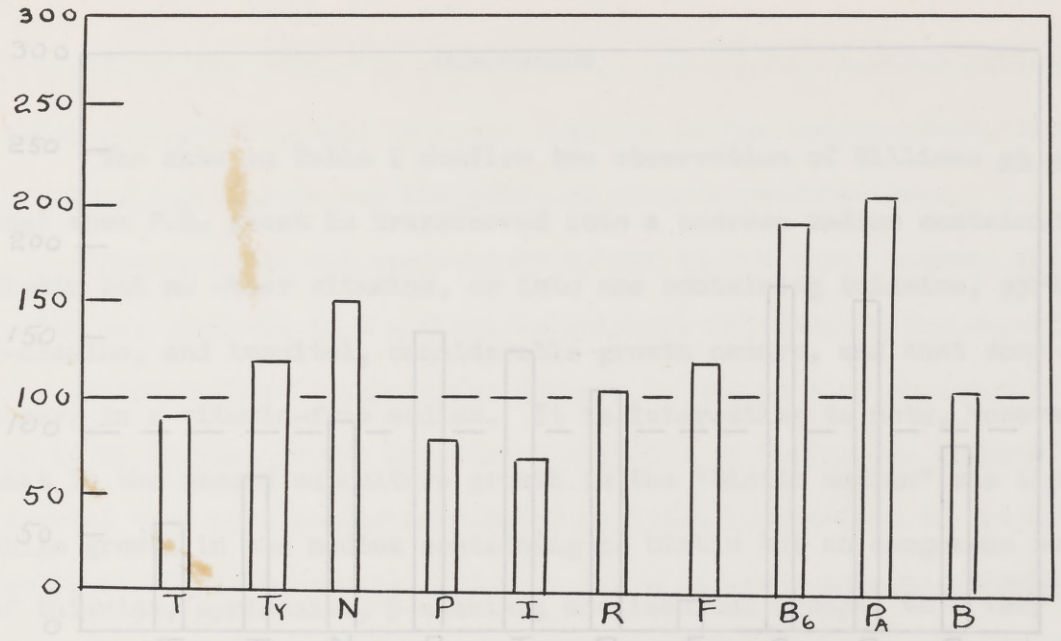

Fig. 13. "Vitamin profile" for yeast from the "biotin medium.

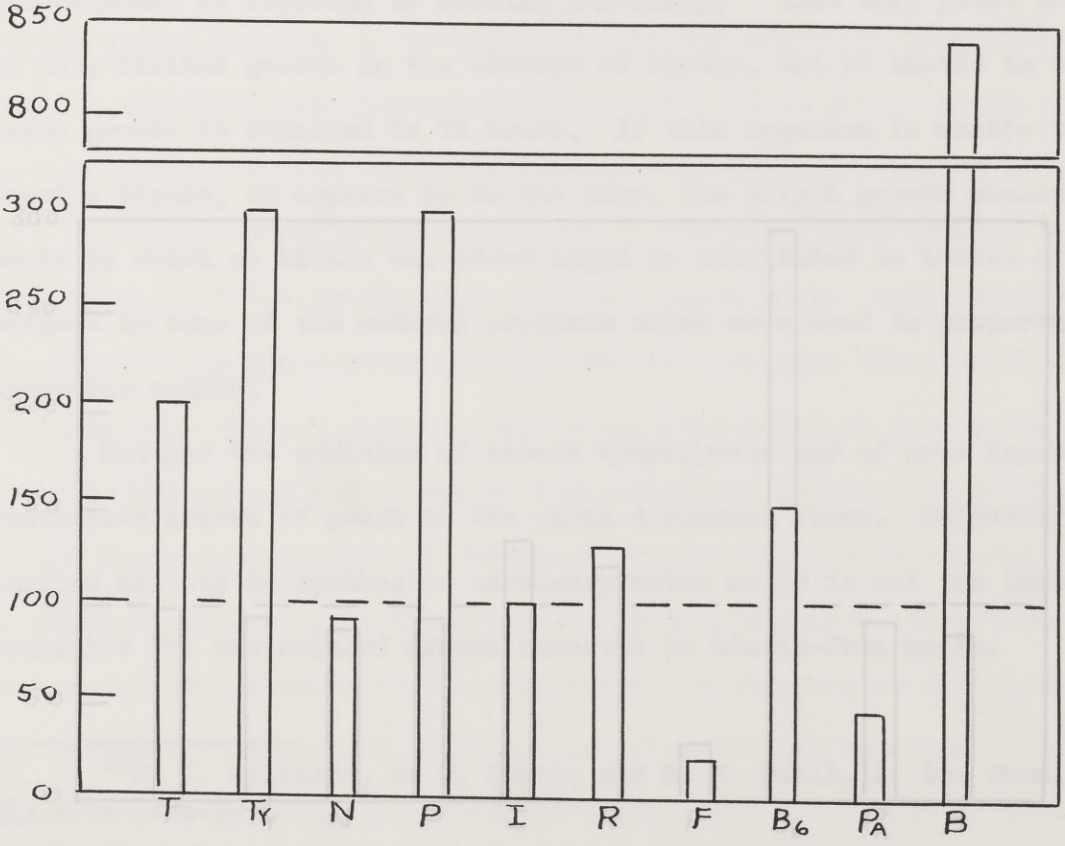

Fig. 14. "Vitamin profile" for galac yeast (crop No. 2).

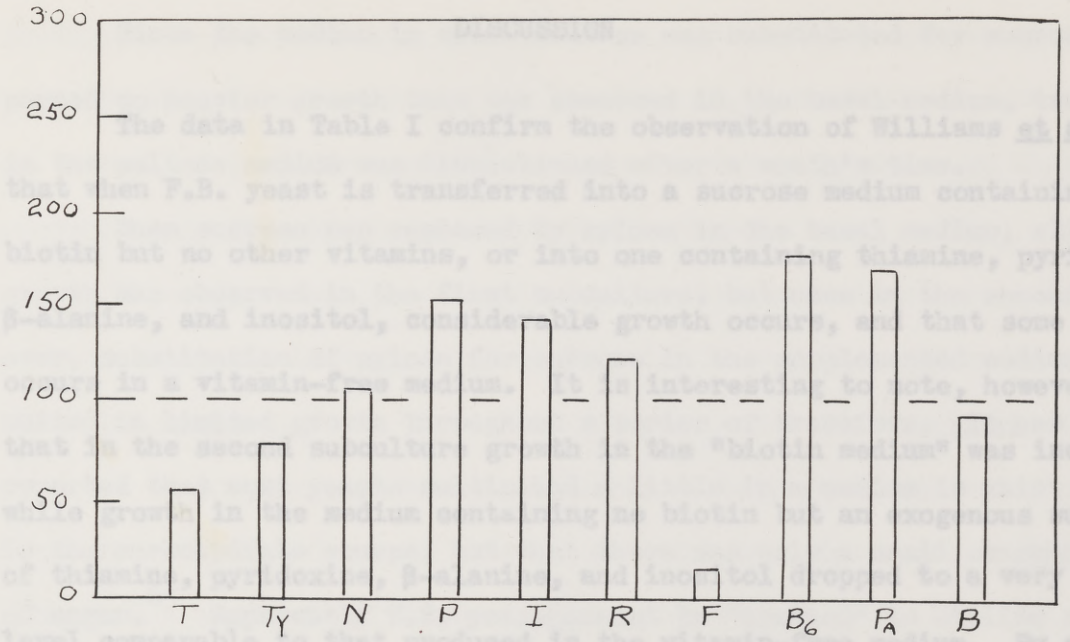

Fig. 15. "Vitamin profile" for galac yeast (crop No. 6).

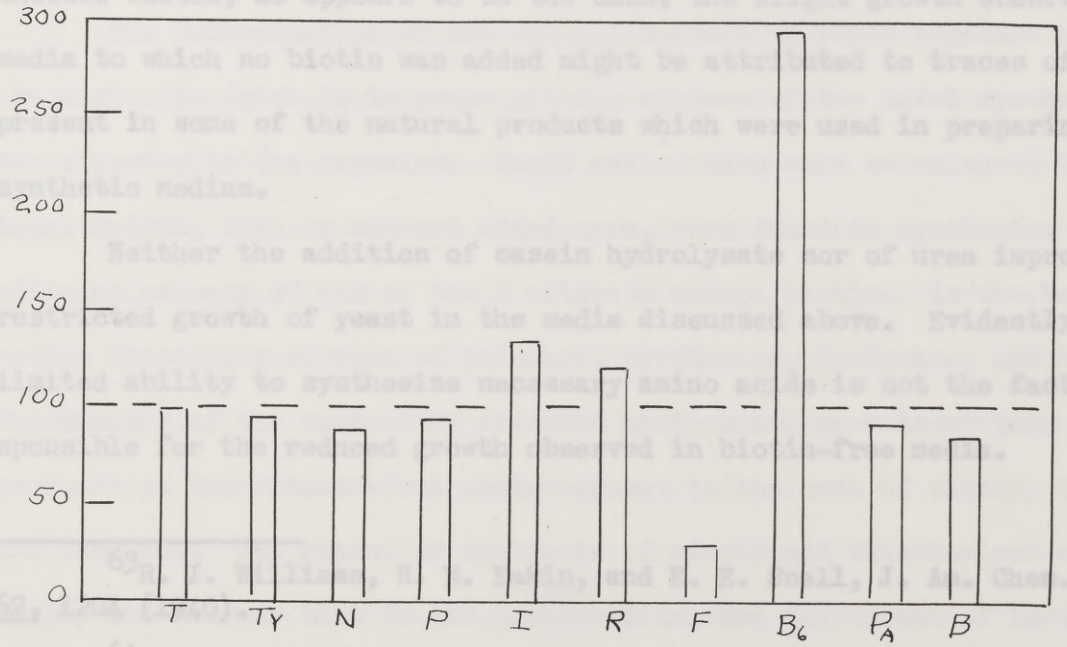

Fig. 16. "Vitamin profile" for galac yeast (crop No. 8).

DISCUSSION

The data in Table I confirm the observation of Williams et al.,[63] that when F.B. yeast is transferred into a sucrose medium containing biotin but no other vitamins, or into one containing thiamine, pyridoxine, β-alanine, and inositol, considerable growth occurs, and that some growth occurs in a vitamin-free medium. It is interesting to note, however, that in the second subculture growth in the "biotin medium" was increased, while growth in the medium containing no biotin but an exogenous supply of thiamine, pyridoxine, β-alanine, and inositol dropped to a very low level comparable to that produced in the vitamin-free medium. By successive subculturing in these media over an extended period of time it was established, as reported by Leonian and Lilly,[64] that this yeast is capable of only limited growth in the absence of biotin, but if biotin is supplied, heavy growth is obtained in 72 hours. If this organism is unable to synthesize biotin, as appears to be the case, the slight growth observed in media to which no biotin was added might be attributed to traces of biotin present in some of the natural products which were used in preparing the synthetic medium.

Neither the addition of casein hydrolysate nor of urea improved the restricted growth of yeast in the media discussed above. Evidently a limited ability to synthesize necessary amino acids is not the factor responsible for the reduced growth observed in biotin-free media.

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[63] R. J. Williams, R. E. Eakin, and E. E. Snell, J. Am. Chem. Soc., 62, 1204 (1940).

[64] L. H. Leonian and V. G. Lilly, J. Bact., 45, 329 (1943).

Since the medium in which maltose was substituted for sucrose supported no heavier growth than was observed in the basal medium, transfer in the maltose medium was discontinued after a month's time.

When sucrose was replaced by xylose in the basal medium, slight growth was observed in the first subculture, but none in the second. However, substitution of xylose for sucrose in the supplemented medium resulted in limited growth throughout a series of transfers. It has been reported that most yeasts multiplied a little in a medium in which xylose is the carbohydrate source, but that there was only a small consumption of sugar.[65] Apparently F.B. yeast cannot be "trained" to utilize xylose satisfactorily even though the acclimatization period is prolonged.

The addition of casein hydrolysate to the vitamin-supplemented xylose medium had some improving effect on the growth of yeast, but a limited ability to synthesize essential amino acids cannot be the only factor responsible for the restricted growth of the yeast in this medium.

The determination of the vitamin content of yeast together with the medium in which it is grown gives a measure of the total synthesis of the vitamins by the organism. Yeast cells which were subcultured in the basal medium, with or without added urea, were found to synthesize significant amounts of all of the B vitamins except biotin. In the basal medium containing addenda of thiamine, pyridoxine, β-alanine, and inositol, the amounts of the respective vitamins synthesized were lower than those produced in the vitamin-free medium except in the case of niacin, thiamine, and inositol. The extent of synthesis of niacin and thiamine was essentially the same as that in the basal medium, but the amount of inositol

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[65] R. Lechner, Vorratspflege u. Lebensmittelforsch., 3, 220 (1940); Chem. Abstr., 36, 6188 (1942).

produced was approximately doubled. The most marked decreases were observed in p-aminobenzoic acid and vitamin B_6 formation. Apparently one or several of the added nutrilites suppresses the formation of these substances or is able to replace them in function.

Since the yeast crops in the media discussed above were only one-seventh to one-twelfth as large as that in the supplemented medium, while the total amounts of the respective vitamins synthesized were approximately as great or greater than those contained in the yeast crop separated from the supplemented medium, it appears probable that in no case is the entire amount of a synthesized vitamin retained by the cells.

In the supplemented medium in which xylose was substituted for sucrose, the amounts of the B vitamins found were sufficient to indicate that some synthesis of every one except biotin and vitamin B_6 had occurred. No values were obtained for the latter because the extracts were toxic to the test organism. In every case except that of thiamine, the amounts were lower than those produced in the basal medium, folic acid and p-aminobenzoic acid being especially reduced in amount. The addition of casein hydrolysate to the xylose medium increased the extent of growth approximately 33 per cent and caused a concomitant increase in the synthesis of niacin, riboflavin, folic acid, thiamine, and p-aminobenzoic acid.

The inositol values listed in Table II were determined on both acid and enzyme extracted samples. Inspection of the values suggests that most of the inositol which is synthesized by the yeast is bound in such a fashion that acid hydrolysis is required for its liberation. In the xylose medium there is no appreciable difference between inositol values for enzyme-digested and for acid-digested samples, so that any inositol

synthesized from this pentose must be combined in a form different from that produced in the other modified media.

Since it has been shown that F.B. yeast is capable of synthesizing appreciable amounts of all of the B vitamins except biotin, it is not surprising that the vitamin content of the "biotin yeast" is very like that of the control yeast. The "profile" for "biotin yeast" (Fig. 13) shows that this yeast is able to meet its vitamin requirements by synthetic activity, and indicates that its requirement for pantothenic acid, riboflavin, thiamine, and folic acid must be very like that of the control. A lowered inositol content is observed, and increased amounts of niacin, vitamin B_6, and p-aminobenzoic acid are present. The fact that increased amounts of the last two nutrilites named are contained in the "biotin yeast" is in accord with the observation made in determining the amounts of vitamins synthesized in the qualitatively modified media; the presence of thiamine, pyridoxine, β-alanine, and inositol in the culture medium causes a reduction both in the amounts of vitamin B_6 and p-aminobenzoic acid synthesized by yeast and in the amounts contained in the cells. Further investigation is necessary in order to determine which of the four added vitamins are responsible for these reduced contents, and what is the significance of the changes.

The fact that the crops of galac yeast were prepared in a medium supplemented with yeast extract, a rich source of the B vitamins, makes it impossible to draw any conclusions concerning the synthetic activity of this derived yeast. But if cell content is a fair index of the requirement for a given vitamin, it is clear that the galac yeast requires larger amounts of vitamin B_6 than does the parent strain. Inspection of the graphs

for the three crops assayed (Figs. 14-16) reveals a progressive increase in the amount of vitamin B_6 contained by the yeast, the amount in crop No. 8 being approximately three times that in the control organism.

The acclimatization of F.B. yeast in the galactose medium was accompanied by no significant change in niacin content, while the riboflavin content was uniformly slightly higher and the folic acid content consistently lower in the galac yeast than in the control. Crops Nos. 6 and 8 showed slightly increased inositol contents. Certain eccentricities which cannot be satisfactorily explained were observed in the pantothenic acid, thiamine, biotin, and p-aminobenzoic acid contents of the three crops. If crop No. 8 represents a yeast which is predominately a galactose fermentor, and this certainly seems probable, the pantothenic acid, thiamine, biotin, and p-aminobenzoic acid contents of galac yeast are essentially the same as those of the parent yeast.

In making a critical study of the changes in the vitamin content of yeast cultured in the presence of inhibitors, it is necessary to recognize that an observed lowering in content may be interpreted as arising from (1) a diminished need for, (2) an interference in the synthesis of, (3) a lessened retention of, or (4) in some cases, a reduced absorption from the medium of that vitamin. An observed increase may be regarded as evidence for an enhanced requirement, a stimulation of synthesis, or as the result of increased retention of the vitamin by the cells.

It has been established that F.B. yeast is capable of synthesizing considerable amounts of all of the B vitamins except biotin, so that probably absorption effects need not be considered. Further, under standardized conditions of culture and harvesting it seems likely that the

vitamin content of the yeast cells will reflect the need for the nutrilites, and from this point of view the extent of variation in content of the respective vitamins and the significance of these changes will be considered.

Thiamine and Niacin--Since there was a fair degree of parallelism between thiamine and niacin values, particularly in those cases in which the contents were unchanged or lowered, they will be considered together. The thiamine values referred to in the following discussion are those obtained with Streptococcus salivarius as the test organism, since the yeast assay method has been shown not to be completely specific for thiamine.[66] Although yeast growth assay values were consistently higher (2 to 4 times) than corresponding values from the Streptococcus salivarius test (Table III), inspection of the "profiles" in Figs. 1-12 reveals that when assay values are expressed in terms of percentage content of the control yeast, for seven of the yeast crops produced in the presence of inhibitors the difference between the heights of the two thiamine bars of each is less than 10 per cent. For the other four crops the yeast growth values are as much as 30 per cent higher. This agreement between the two sets of assay values, one of which represents thiamine, the other of which represents thiamine and some other active substance, possibly the thiazole moiety, indicates that decreases or increases in thiamine content are attended by corresponding changes in content of a non-thiamine material measured in the yeast growth method. This stimulative non-thiamine material may be regarded as a precursor of the vitamin or as a degradation product.

It has been shown that the thiamine content of Saccharomyces cerevisiae and other yeasts increases in anaerobic culture and that the

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[66] M. A. Eppright and R. J. Williams, Ind. Eng. Chem., Anal. Ed., 16, 576 (1944).

fermentation energy increases with increased thiamine content.[67] It seems reasonable to assume that thiamine content may be used to determine the extent to which various inhibitors interfere with or enhance fermentation, as may the correlative niacin content.

On this basis, propamidine, urethane, and camphor (0.001 M) had no effect on the course of fermentation since the thiamine and niacin contents were essentially those of the control yeast; chloral hydrate caused a slight reduction in content so that its effect on fermentation appears to be negligible also. The reagents for the carbonyl group, viz., potassium cyanide, sodium sulfite, semicarbazide, and hydroxylamine, caused considerably lowered contents of these two vitamins as was to be expected. With semicarbazide the lowering was most marked, but this reagent was used at a concentration 10 times as high as the other three keto-fixatives. Sodium fluoride also caused a sharp reduction in thiamine and niacin values, which was entirely expected inasmuch as it is known to inhibit two of the enzymes, enolase and carboxylase, active in the fermentation chain of reactions, and to prevent the formation of cozymase, which should result in a reduced demand for nicotinamide. Yeast from the sulfaguanidine and saturated camphor media had niacin contents considerably higher than the corresponding thiamine contents. This may mean that niacin functions in processes other than the oxidation-reduction changes in fermentation, or it may be that these inhibitors alter cell retention of these vitamins differently.

Pantothenic Acid--In only one case, viz., in the "cyanide yeast", was an increase of more than 20 per cent in pantothenic acid content

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[67] E. N. Odintsova, Microbiology (U. S. S. R.), 10, 670 (1941); Chem. Abstr., 38, 2685 (1944).

observed. The other carbonyl reagents did not cause similar increases; hydroxylamine caused an increase of approximately 18 per cent, but sodium sulfite and semicarbazide caused a reduction in content of 75 to 90 per cent. Evidently the action of cyanide on the pantothenic acid content of yeast relates to some property other than its ability to combine with the carbonyl group. Since a sharp decrease (more than 85 per cent) in content was observed in "urethane yeast", and since urethane is a recognized dehydrogenase inhibitor, it is conceivable that pantothenic acid acts in a dehydrogenase system which is put out of operation by urethane and is called into increased activity in the presence of cyanide, which blocks out the cytochrome system operative in respiration. An alternate interpretation is that a dehydrogenase system is involved in the formation of pantothenic acid.

The absence of any correlation between changes in pantothenic acid content and in thiamine and niacin values makes it appear that most probably the chief metabolic role of pantothenic acid does not reside in the fermentation chain of reactions. It has been reported that the addition of pantothenic acid (or β-alanine) to Saccharomyces cerevisiae grown in the absence of pantothenic acid caused a primary strong acceleration in respiration and a slight inhibition of fermentation.[68, 69] On the other hand, Williams and coworkers found that the addition of pantothenic acid to yeast which was grown in a pantothenic acid deficient medium caused a marked increase in fermenting ability[70] accompanied by a binding of

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[68] V. Hartelius, Naturwissenschaften, 30, 660 (1942).

[69] Ibid., 31, 440 (1943).

[70] E. F. Pratt and R. J. Williams, J. Gen. Physiol., 22, 637 (1939).

pantothenic acid by the cells.[71] In a study of Proteus morganii grown in suboptimal amounts of pantothenic acid, it was shown that the addition of pantothenic acid caused practically no change in the extent of fermentation of glucose, but did cause an increased aerobic metabolism of pyruvic acid.[72] The part played by pantothenic acid in cell metabolism remains obscure; some of the evidence points to its involvement in respiratory processes, some to a function in fermentation reactions.

With chloral hydrate, propamidine, and camphor (0.001 M), the pantothenic acid content was approximately 80 per cent that of the control yeast, which indicates that these reagents must not seriously affect the need of the cells for this nutrilite. In the yeast from the saturated camphor and the sulfaguanidine media, the pantothenic acid content dropped to 60 per cent and 46 per cent, respectively, of the value for the control. Fluoride also caused a lowered pantothenic acid content, though the reduction was not as drastic as that observed for several of the other vitamins.

Inositol--Of the inhibitors employed, only propamidine caused the inositol content of yeast to fall as low as 50 per cent of the value for the control yeast, and only sulfaguanidine caused an increased content in this vitamin. There is some parallelism between changes in inositol values and corresponding changes in thiamine and niacin contents which suggests that inositol may also be involved in fermentation reactions. Previously, in a study of the vitamin content of rat tissues, mathematical coefficients were derived for the correlation between respective vitamin contents and

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[71] P. C. Teague and R. J. Williams, ibid., 25, 777 (1942).

[72] G. M. Hills, Biochem. J., 37, 418 (1943).

corresponding data for oxygen consumption and for anaerobic glycolysis; only inositol and biotin were found to show a positive correlation with both aerobic respiratory rate and anaerobic glycolysis rate.[73] If inositol functions in both aerobic and anaerobic processes in yeast, it is not surprising that the cells of the organism maintain a relatively constant amount of the substance even though they are grown under adverse conditions.

Riboflavin—Two of the carbonyl reagents used as inhibitors, hydroxylamine and sulfite, caused no appreciable changes in riboflavin content, while semicarbazide caused a reduced content and cyanide an increased one. The apparent lack of consistency in changes in riboflavin values for yeast cultured in the presence of these keto-fixatives indicates that this vitamin probably is not directly involved in the reactions occurring in fermentation. Other evidence for the non-participation of riboflavin in fermentation changes has been obtained by Williams,[74] who showed that the riboflavin content of rat tissues had a significant positive correlation with aerobic respiration but a negative correlation with anaerobic glycolysis.

An increased content of riboflavin in bakers' yeast cultured in the presence of cyanide has been observed previously by Pett,[75] who also found that other reagents such as cysteine and pyridine, which can form complexes with iron-containing enzymes, increased the amount of riboflavin in yeast. He suggested that respiration in yeast may take place along two paths, one

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[73] R. J. Williams, Vitamins and hormones, New York, 1, 229 (1943).

[74] Ibid.

[75] L. B. Pett, Biochem. J., 30, 1438 (1936).

of which involves the cytochromes and one in which a flavoprotein is active; the latter system, being cyanide insensitive, can account for the residual respiration often observed in the presence of cyanide as well as for the lack of any suppressive effect of the inhibitor on the respiration of "cyanide yeast". Some supporting evidence for this hypothesis has been obtained by Stier and Castor[76] who prepared a "cyanide substrain" of Saccharomyces cerevisiae which lacked cytochrome activity and appeared to lack certain components of the enzyme system responsible for most of the oxygen consumption in the parent strain.

If riboflavin actually participates in such an auxiliary respiratory system in yeast, changes in the amount of the substance contained in the cells should serve as a measure of the extent of activity of this secondary system. From this standpoint, sulfaguanidine and camphor (0.001 M) must increase the activity of the flavin system since both caused increases in riboflavin content of the same order of magnitude as that obtained with cyanide. Propamidine, urethane, chloral hydrate, fluoride, and camphor (saturated) caused reductions in riboflavin content, the effect of fluoride being the most acute. The action of fluoride responsible for the diminished riboflavin content probably relates to its property of phosphatase inhibition.

Folic Acid--No correlation was observed between changes in folic acid content and changes in the other nutrilites. Only in one instance was the content of this vitamin increased, and that was in the presence of sulfite. Even though the other keto-fixatives produced a lowered folic acid content in the yeast, the general lack of correlation between changes

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[76] J. B. Stier and J. G. B. Castor, J. Gen. Physiol., 25, 229 (1941).

in folic acid and thiamine-niacin values makes it appear that folic acid is probably not involved directly in the fermentation reactions. Two of the reagents, urethane and propamidine, neither increased nor decreased the folic acid content, and all of the other reagents reduced its content considerably, fluoride causing the content to fall to only 4 per cent of that of the control yeast.

In the yeast crops grown in the two camphor media, the difference in folic acid content was greater than that in any other vitamin present. This, however, does not explain the fact that the more concentrated camphor medium caused the development of involution in the yeast since other yeasts had similar folic acid contents but did not exhibit pleomorphism.

The folic acid values obtained may not be an actual measure of the total combined folic acid in yeast; so any deductions based on these values cannot be defended. Recently, Totter et al.[77] found that the apparent folic acid content of brewers' yeast was increased approximately 15 times by incubating the yeast with fresh liver preparations rather than with clarase.

Vitamin B_6--The values for vitamin B_6 probably represent pyridoxine, pyridoxal, and pyridoxamine since the test organism, Saccharomyces carlsbergensis, used in the assay responds equally to these three active substances.[78]

Two of the keto-fixatives, sulfite and hydroxylamine, had essentially no effect on the vitamin B_6 content of yeast. With cyanide, the content

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[77] J. R. Totter, V. Mims, and P. L. Day, Science, 100, 223 (1944).

[78] E. E. Snell, J. Biol. Chem., 154, 313 (1944).

of the yeast from the shaken culture was the same as that of the control yeast, but the content of the yeast from the unshaken culture was approximately 25 per cent lower. Though semicarbazide caused a lowering in content of very nearly 80 per cent, it seems probable that vitamin B_6 is not involved in the fermentation processes of yeast since changes in this vitamin show no significant correlation with changes in thiamine and niacin values.

The use of chloral hydrate and sulfaguanidine as inhibitors resulted in a more than doubled vitamin B_6 content, while urethane, propamidine, and camphor produced lowered values. It is significant that yeast cultured in the presence of fluoride was quite low in every vitamin except vitamin B_6, biotin, and inositol. Since this reagent inhibits fermentation at two sites and is also an inhibitor of yeast phosphatases, it seems likely that vitamin B_6, as well as biotin and inositol, functions in processes which are not linked with the action of phosphatases and which are not part of the normal fermentation chain of reactions. Snell[79] has suggested that pyridoxamine and pyridoxal may function in a hydrogen transport system or play a part in transamination processes. The reduction in vitamin B_6 content observed with urethane, a dehydrogenase inhibitor, might be considered as indirect evidence for the first type of activity. It may indicate, however, that dehydrogenases are essential for synthesis of this vitamin.

p-Aminobenzoic Acid--No significant degree of correlation between changes in content of p-aminobenzoic acid and any other vitamin was

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[79] Ibid.

observed. That variations in this vitamin content do not generally parallel corresponding trends for thiamine and niacin indicates a probable nonparticipation of the nutrilite in fermentation reactions. The carbonyl reagents, which uniformly suppressed thiamine and niacin contents in yeast, did not have a similarly consistent effect on p-aminobenzoic acid values. Both hydroxylamine and semicarbazide caused considerably reduced values, sulfite and cyanide (in the unshaken culture) increased ones.

The "fluoride yeast" contained only 12 per cent as much p-aminobenzoic acid as was in the control yeast, which may mean that the formation of p-aminobenzoic acid, or its action in yeast, depends on reactions involving fluoride-sensitive phosphatases.

The yeast crop from the sulfaguanidine medium was only 35 per cent low in p-aminobenzoic acid, indicating that this inhibitor did not markedly block synthesis of the vitamin even though its interference with the function of p-aminobenzoic acid was to be expected because of structural similarity.

The other inhibitors, with a single exception, had virtually no effect on the amount of p-aminobenzoic acid present in yeast. Yeast from the camphor (saturated) medium had a reduced content of this vitamin.

Biotin--In no case did the biotin content of any yeast fall below that of the control yeast; this serves as further evidence that F.B. yeast must contain within its cells a limiting level of biotin in order to carry on those reactions essential for extensive growth.

In a study of the role of biotin in fermentation, respiration, growth, and nitrogen assimilation of yeast, Winzler et al.[80] showed that

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[80] R. J. Winzler, D. Burk, and V. du Vigneaud, Arch. Biochem. 5, 25 (1944).

both fermentation and respiration rates of biotin-deficient yeast were much lower than in normal yeast. The addition of biotin to such a yeast caused an almost immediate increase in the aerobic ($Q^{O_2}_{CO_2}$) as well as the anaerobic ($Q^{N_2}_{CO_2}$) rate of fermentation. The increase in fermentation was followed by an increase in respiration (Q_{O_2}), and this in turn was followed by an increase in growth. All of these changes were observed only provided assimilable nitrogen was available. These investigators concluded that biotin is more intimately linked with fermentation processes than with those of respiration and growth.

In a number of cases the biotin content of yeast was increased by culturing in the presence of inhibitors; in some instances, notably with sulfite, semicarbazide, propamidine, urethane, chloral hydrate, and camphor (0.001 M), this increase can be accounted for without assuming any synthesis of the vitamin occurs, since the biotin available in the medium is sufficient to account for the increase. In the yeast from the cyanide medium which was unshaken, the biotin content was high enough to make it appear that some synthesis occurred, as was the case with yeast from the fluoride, hydroxylamine, camphor (saturated), and sulfaguanidine media. The most striking increase was observed in yeast from the sulfaguanidine medium; here the biotin content was more than 4 times that of the control yeast and amounted to more than twice the quantity of biotin which was added to the culture medium. The possibility was considered that the guanidine group might serve as a biotin precursor, but an attempt to obtain a yeast capable of extensive growth in a sulfaguanidine medium from which biotin was omitted was unsuccessful.

SUMMARY

The problem of how most of the B vitamins function and the extent of interrelations between their functions is tremendously complex, and its solution must await further investigation. In yeast crops which were grown in the presence of various inhibitors, there was considerable variation in content of the various B vitamins. A higher degree of parallelism in variation in content was found to exist between thiamine and niacin than between any other pair of vitamins; this has been interpreted as indicating that the predominant functions of these two vitamins are their established roles in fermentation. The values for inositol indicate that it may be involved in fermentation processes, but this is not the case for the other members of the B complex.

Of the vitamins studied, biotin appears to be unique since in no case did the biotin content of any yeast produced in the presence of an inhibitor fall below that of the control yeast. There was some evidence of synthesis of biotin, or some material with biotin activity, in the presence of certain inhibitors, the most striking instance being with sulfaguanidine. However, an attempt to culture F.B. yeast in a biotin-free sulfaguanidine medium was unsuccessful.

An exogenous supply of biotin was essential for extensive proliferation of F.B. yeast, and yeast grown in a medium to which biotin was the only added vitamin contained the B vitamins in amounts very similar to those found in yeast grown in a vitamin-supplemented medium, the most marked differences being in increased vitamin B_6 and p-aminobenzoic acid contents. In the absence of biotin, significant amounts of all of the B vitamins except biotin were synthesized, both in the presence and absence

of certain other members of the B complex. The presence of thiamine, pyridoxine, inositol, and β-alanine in the culture medium caused a reduction both in the amounts of vitamin B_6 and p-aminobenzoic acid synthesized and in the amounts of these two nutrilites contained in the cells of yeast cultured in such a medium.

F.B. yeast was able to grow in a xylose medium only when certain of the B vitamins were present, and even then growth was limited. Evidence was obtained, however, for some synthesis of all of the vitamins investigated except biotin and vitamin B_6 in the xylose medium.

The most significant differences in vitamin content between galac yeast and the parent F.B. strain were in folic acid and vitamin B_6, the former being considerably reduced in amount, the later being increased.

PART II

FACTORS NOT SYNTHESIZED IN THE PRESENCE OF CYANIDE

INTRODUCTION

In Part I of this dissertation it was pointed out that, in order to obtain a crop of the "cyanide yeast" for assay, it was necessary to extend the incubation period beyond the 72 hour interval used with the other inhibitors. This difficulty was unexpected since other investigators,[81, 82] who have cultured Saccharomyces cerevisiae in a cyanide medium containing the same concentration of the inhibitor, have reported no difficulties in obtaining growth. However, a synthetic medium was employed in the current investigation whereas a malt extract medium[83] and a medium supplemented with marmite,[84] a yeast extract preparation, were used in the earlier studies. This fact suggested that these ingredients supplied one or more substances which the yeast required for growth but which it was unable to synthesize in the presence of cyanide at a rate which would permit rapid growth.

Preliminary experiments were performed which showed that yeast extract and certain other natural materials had an anti-cyanide activity which could not be duplicated by any of the known compounds tested. Leonian and Lilly[85] have reported that the effect of yeast extract in improving the

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[81] L. B. Pett, Biochem. J., 30, 1438 (1936).

[82] J. B. Stier and J. G. B. Castor, J. Gen. Physiol., 25, 229 (1941).

[83] Ibid.

[84] L. B. Pett, op. cit.

[85] L. H. Leonian and V. G. Lilly, J. Bact., 45, 191 (1943).

growth of F.B. yeast* is not due to some unknown substance but to the fact that the extract increases the levels of inositol, biotin, and pantothenic acid; these investigators used a culture medium which was supplemented with a casein hydrolysate. In the cyanide medium employed in the present study, addition of higher concentrations of these vitamins was without effect, although in some later experiments the addition of increased amounts of pantothenic acid (or β-alanine) had some beneficial action.

Further studies on the nature of the active principle in yeast extract responsible for the reversal of cyanide inhibition of growth led to the conclusion that at least two substances other than pantothenic acid were involved. These two materials were separated by adsorption and elution procedures.

Attempts to identify the resulting filtrate and eluate principles with known compounds were not successful. However, some information concerning their properties has been obtained.

The details of experimentation together with the implications of the results obtained will be presented in the following sections.

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*The yeast which Leonian and Lilly[86] studied was isolated by them from Fleischmann's yeast and carries the same strain designation as the yeast used in the present investigation.

[86] L. H. Leonian and V. G. Lilly, J. Bact., 45, 191 (1943).

PRELIMINARY EXPERIMENTS

As a means of determining whether or not yeast extract would reverse the inhibitory effect of cyanide on yeast growth, increasing amounts (0 to 20 mg.) of Difco yeast extract were added to a series of 50 ml. Erlenmeyer flasks, each of which contained 20 ml. portions of cyanide medium, and the flasks were inoculated with F.B. yeast. At the end of 24 hours, graded increases in turbidity were noted which seemed to confirm the supposition that some material stimulative to growth was supplied by the extract.

Testing Procedure

Initially, the effect of yeast extract was considered to be due to the activity of a single substance, and a method for quantitative study of the active principle was devised.

The procedure employed was essentially the same as that used in microbiological assay methods. The single innovation introduced was the addition of a growth inhibitor, potassium cyanide, to a synthetic medium which normally supported heavy growth of the test organism. The resulting cyanide medium was deficient with respect to some growth-promoting substance present in the selected standard extract, which meant that the choice of an arbitrary unit of activity for the standard material permitted quantitative measurement of the activities of various known compounds and natural extracts.

Cyanide Medium---The medium first used was identical with the vitamin-supplemented medium described in Part I (p. 18) except for the addition of 78 mg. per liter of potassium cyanide. This amount of cyanide gave a

concentration of 0.0012 M, but the concentration was adjusted finally to 0.001 M by dilution with appropriate volumes of standard or sample solutions. A weighed portion of the inhibitor was added to the sterile medium immediately before inoculation with yeast, preparatory to adding the seeded cyanide medium to the tubes.

Later in the investigation, pantothenic acid (2 mg. per liter) was substituted for β-alanine in the cyanide medium, and cysteine (20 mg. per liter) was also added as a supplement.

Inoculum--In order to prepare an inoculum, F.B. yeast was transferred from an agar slant into 20 ml. of sterile supplemented medium contained in a 50 ml. Erlenmeyer flask and incubated for 24 hours at 30° C. An aliquot of the resulting suspension containing 0.4 mg. of moist yeast was added per 100 ml. of cyanide medium.

Standard Material--A sample of Difco yeast extract, lot No. 335368, was the standard material selected. The growth-promoting activity of 1 mg. of this extract was defined as 1 mg. unit. Often it was convenient to use the term "potency" as an expression of the relative effectiveness of a material in terms of yeast extract, i.e., to indicate apparent mg. units per mg. of the material assayed.

Growing of Cultures--Assays were carried out in 4 inch test tubes of uniform diameter supported in a wire rack. Solutions of the standard material and substances to be tested were pipetted into the tubes in volumes up to 1 ml.; when less than the limiting volume was used, distilled water was added to bring the volume in each tube to 1 ml. Sterilization of the tubed materials was unnecessary since the cyanide present in the added medium served to prevent contamination under the conditions used. Five ml.

portions of the seeded cyanide medium were added to each tube and the rack was shaken to insure suitable mixing of samples and medium. A folded towel was placed over the tubes, and the rack was placed in a water bath at 30° C. for 15 to 16 hours. At the end of the incubation period, the extent of growth was measured turbidimetrically, and galvanometer readings were plotted against corresponding mg. units of the standard material. Thus the activity of other materials could be calculated in terms of the standard.

Table IV shows the growth response of F.B. yeast to various levels of yeast extract under test conditions.

TABLE IV

Growth Response of F.B. Yeast to Yeast Extract in Cyanide Medium

Amount of yeast extract added	Galvanometer reading
mg. per tube	
0	5
0.2	11
0.5	22.5
1.0	32
5.0	62
10.0	81

Activity of Various Known Compounds

After establishing suitable testing conditions, the activity of a number of known compounds was determined in order to ascertain whether or not the anti-cyanide action of yeast extract depended on the presence of a familiar substance such as a known vitamin or amino acid.

The following compounds were tested separately and found to be inactive: riboflavin, niacin, p-aminobenzoic acid, choline, guanine, uracil, thymine, xanthine, 5-carboxyuracil, adenine, adenosine, adenylic acid, glycine, alanine, valine, leucine, isoleucine, threonine, serine, hydroxyproline, proline, arginine, lysine, histidine, phenylalanine, glutamic acid, cystine, glutathione, taurine, creatinine, ascorbic acid, propionic acid, succinic acid, formic acid, malic acid, lactic acid, fumaric acid, pyruvic acid, sodium thioglycolate, 4-amino-2-methyl-1-naphthol, and 2-methyl-1,4-naphthoquinone. The amino acids were tested at a level of 1 mg. per tube and all of the other compounds at concentrations up to 100 γ. A concentrate of folic acid was tested and found to be inactive at levels representing as much as 25 γ of folic acid* per tube.

The effect of increasing the concentration of certain substances which were present in the test medium was determined. Thiamine (100 γ), pyridoxine (100 γ), inositol (1 mg.), biotin (0.2 γ), aspartic acid (1 mg.), β-alanine (100 γ), and ferric chloride (100 γ) were each found to be ineffective when added to individual cultures.

Various mixtures of compounds when added to individual cultures also proved to be inactive. Each of the following mixtures had no anti-cyanide activity: (1) adenine, guanine, and uracil, 100 γ of each; (2) thiamine, 20 γ, pyridoxine, 20 γ, inositol, 5 mg., pantothenic acid, 100 γ, and biotin, 1 γ; (3) thiamine, 10 γ, pyridoxine, 10 γ, pantothenic acid, 10 γ, biotin, 0.1 γ, riboflavin, 10 γ, and folic acid, 25 γ.

*All values for folic acid which appear in this dissertation are in terms of a material of "potency 40,000", as defined by Mitchell and Snell.[87]

[87] H. K. Mitchell and E. E. Snell, Univ. Texas Pub., 4137, 36 (1941).

Of all of the compounds tested, only methionine and cysteine were found to have any activity. They did not in any sense, however, duplicate the effect of yeast extract; this fact is evident from the results in Table V which include the respective potencies measured at two levels and the potency of a mixture of the two amino acids.

TABLE V

Anti-Cyanide Activity

of Methionine and Cysteine

Substances tested	Amount added	Apparent potency
	mg.	
Methionine	1	0.7
	10	0.07
Cysteine	1	0.65
	10	0
Methionine + cysteine (1:1 mixture)	2	0.33

Certain of the amino acids found to be inactive in the cyanide medium have been reported to increase the growth of yeast under other conditions. Mitchell and Williams[88] found that glutamic acid, asparagine, aspartic acid, arginine, leucine, and lysine had the greatest individual stimulatory effects on the growth of F.B. yeast; these investigators used a synthetic culture medium which contained no aspartic acid (or asparagine). Methionine, which stimulates yeast growth in the presence of cyanide, has been reported to have an "anti-biotin" activity; when this amino acid together with biotin was added to Saccharomyces cerevisiae

[88] H. K. Mitchell and R. J. Williams, Biochem. J., 34, 1532 (1940).

which had been cultured in a biotin-free medium, the normal improving effect of biotin on growth was inhibited.[89] In the biotin-free medium, however, added methionine facilitated growth. Nielsen[90] found that methionine had an inhibitory effect on yeast growth in a medium containing biotin unless thiamine and β-alanine were also present.

If the "anti-biotin" action of methionine is related to the action of this amino acid in increasing riboflavin synthesis[91] and to a concurrent development of a flavin-containing auxiliary respiratory system that has been postulated as functioning in yeast,[92] the action of methionine in reversing the cyanide inhibition of growth may be partially explained. It is possible that this amino acid is utilized in the synthesis of an essential protein which operates in the flavin system. A number of enzymes, dehydrogenases in particular, which function in carbohydrate metabolism have been shown to contain sulfhydryl groups which are essential for their action,[93] and methionine may serve as a source of these necessary groups.

It is probable that cysteine acts in a manner somewhat similar to methionine since certain reactions in which it may be involved are inhibited by cyanide. Keilin[94] found that cysteine was rapidly oxidized by cytochrome oxidase in the presence of cytochrome c without a specific dehydrogenase being present but that the oxidation was strongly inhibited by cyanide.

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[89] C. V. Fisher and G. J. Martin, J. Bact., 45, 33 (1943).

[90] N. Nielsen, Biochem. Z., 307, 187 (1941).

[91] P. R. Burkholder, Proc. Nat. Acad. Sci., 29, 166 (1943).

[92] L. B. Pett, Biochem. J., 30, 1438 (1936).

[93] E. S. G. Barron and T. P. Singer, J. Biol. Chem., 157, 221 (1944).

[94] D. Keilin, Proc. Roy. Soc. London, Series B, 106, 418 (1930).

It has been shown that bakers' yeast forms small amounts of hydrogen sulfide from cysteine,[95] but the enzyme, cysteine desulfurase, which converts cysteine into hydrogen sulfide, ammonia, and pyruvic acid has been found to be poisoned by cyanide.[96] The fact that cysteine was found to have no anti-cyanide action at a level of 10 mg. while methionine had the same growth promoting effect at both 1 and 10 mg. levels indicated that their action is not entirely the same.

Activity of Various Source Materials

Since none of the known compounds tested was found to compare with yeast extract in reversing the cyanide inhibition of yeast growth, various biological materials were assayed in order to determine the most suitable source for use in concentration procedures. The activities of the following materials were measured: brewers' yeast extract, malt extract (Difco), powdered dehydrated grass juice (Cerophyl Laboratories), powdered evaporated milk (Dryco), a fermentation residue (Curbay), defatted wheat germ (viobin), liver fraction B (Wilson's), an enzymatic digest of casein (amigen), and an acid-hydrolysed casein preparation (casamino acids). In Table VI the comparative activities of the various materials assayed are presented.

Difco yeast extract and amigen were the most active substances tested. Casamino acids, unlike yeast extract and amigen, produced a relatively constant growth stimulation when added in amounts ranging from 5 to 50 mg. per tube. The possibility was considered that the action of yeast

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[95] Y. Garreau, Compt. rend. Soc. biol., 137, 176 (1943).

[96] F. Binkley, J. Biol. Chem., 150, 261 (1943).

extract might be due to the presence of at least two substances, one of which was also contained in casamino acids and which might or might not be methionine.

TABLE VI

Anti-Cyanide Activity of Various Source Materials

Source material	Apparent potency
Yeast extract (Difco)	1.0 (Standard)
Yeast extract (brewers')	0.20
Malt extract	0.01
Grass juice	0.07
Evaporated milk	Inactive
Fermentation residue	0.20
Viobin	Inactive
Liver fraction B	0.09
Amigen	0.94
Casamino acids	0.07*

*This value represents the apparent potency of casamino acids at a level of 10 mg. per tube; at a level of 1 mg., the apparent potency is 0.7.

EVIDENCE FOR THE OCCURRENCE OF TWO UNKNOWN FACTORS

If the anti-cyanide action of yeast extract depended on two factors, one of which was present at a limiting concentration, it seemed likely that the addition of the limiting factor to yeast extract would produce at least an additive increase in activity. For this reason, the following experiments were performed.

Effects of Certain Substances on the Activity of Yeast Extract

Casamino Acids--The cyanide medium was supplemented with casamino acids at a concentration which provided 10 mg. of the hydrolysate per tube, and the growth stimulation caused by yeast extract in the supplemented and unsupplemented media was compared. A synergistic rather than an additive increase in stimulation was observed in the medium containing casamino acids.

Since yeast extract is a relatively rich source of the B vitamins, the possibility was considered that one or several known members of this group of substances might be involved in the effect observed above. Hence, the respective B vitamins were tested separately and variously combined in the medium which contained casamino acids. Only pantothenic acid and mixtures containing pantothenic acid were found to increase the extent of growth in the medium to which the casein hydrolysate was added. This vitamin was as effective at 10 γ per tube as at 100 γ.

The growth stimulation produced by various levels of yeast extract was determined in the following media: (1) the medium containing β-alanine, (2) the β-alanine medium supplemented with casamino acids, (3) a medium

in which β-alanine was replaced by 2 mg. per liter of pantothenic acid, and (4) a pantothenic acid medium to which casamino acids were added. The results of this comparative study are shown in Table VII.

These data show that pantothenic acid does not increase the growth stimulation produced by yeast extract but does enhance the amount of growth obtained with casamino acids, both in the presence and absence of yeast extract. If it is assumed that only two factors, pantothenic acid (present in yeast extract in non-limiting concentrations) and some other substance (contained in both yeast extract and casamino acids), are involved in the reversal of cyanide inhibition of growth, the growth stimulation produced by yeast extract in the pantothenic acid-casamino acids medium should represent an additive effect. However, this was not found to be the case. It appeared, therefore, that at least two factors other than pantothenic acid are involved.

By increasing the concentration of β-alanine, the growth stimulation produced by casamino acids was enhanced; but β-alanine at a level of 100 γ per tube was not as effective as was pantothenic acid at a 10 γ level. It seemed advisable to substitute pantothenic acid for β-alanine in the culture medium; so the medium used in all subsequent tests contained 2 mg. per liter of pantothenic acid.

Methionine and Various Reducing Agents--Since in a preceding experiment methionine and cysteine were shown to have some anti-cyanide activity, their respective effects on the amount of growth obtained when they were added to increasing levels of yeast extract were compared with that of casamino acids. Cysteine was tested at several levels because earlier it had been found to have no anti-cyanide activity at a concentration of 10 mg.

TABLE VII

Anti-Cyanide Activity of Yeast Extract and Casamino Acids, Separately and Combined, in Media Containing β-Alanine or Pantothenic Acid

Amount of yeast extract added	Extent of growth in various media							
	β-Alanine medium		β-Alanine medium + casamino acids*		Pantothenic acid medium		Pantothenic acid medium + casamino acids*	
	Galvanometer readings	Apparent mg. units†	Galvanometer readings	Apparent mg. units	Galvanometer readings	Apparent mg. units	Galvanometer readings	Apparent mg. units
mg.								
0	6	0	23	0.65	6	0	40	2.0
0.5	21	0.5	49	3.0	22	0.6	54	3.8
1.0	28	1.0	56	4.4	28	1.0	59	5.0
5.0	59	5.0	69	> 5	59	5.0	73	> 5

*The concentration of casamino acids was 10 mg. per tube.

†The response to yeast extract alone in the β-alanine medium was used as standard (cf. text).

per tube. Certain reducing agents other than cysteine were also added to yeast extract and the extent of growth in the various mixtures was determined. The following substances were added in turn to portions of the cyanide medium in amounts sufficient to supply the indicated concentration per tube: casamino acids (10 mg.), methionine (1 mg.), cysteine (0.01, 0.02, 0.1, 0.2, 0.5, and 1 mg. respectively), sodium thioglycolate (0.1 mg.), glutathione (0.1 mg.), ascorbic acid (0.1 mg.), hydroquinone (0.01 mg.), and sodium hydrosulfite (0.1 mg.). The results in Table VIII are a measure of the comparative extents of growth in these supplemented media.

The extent of growth produced by yeast extract in the methionine-supplemented medium was greater than that produced in the absence of methionine, but it was not as great as that produced in the medium containing casamino acids. Since the amount of methionine added was more than twice the amount of this amino acid contained in casein,[97] it appeared that the activity of the casein hydrolysate did not depend solely on its content of methionine.

The effect of none of the reducing agents equalled the optimum growth stimulation observed in the medium supplemented with cysteine (20 mg. per liter). In every case except that of hydroquinone, they did cause some enhancement of growth in the presence of yeast extract. It is entirely possible that, by testing each of the reducing agents at a series of levels, respective concentrations might have been found which would equal the effect obtained with cysteine, but this was not further investigated. In all subsequent experiments, however, the cyanide medium was supplemented with 20 mg. per liter of cysteine.

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[97] R. J. Williams, A textbook of biochemistry, New York, 2nd edition, p. 92 (1942).

TABLE VIII

Comparative Effects of Supplementing a Medium Containing Yeast Extract with Various Substances

Amount of yeast extract added	Extent of growth* in the presence of various supplements												
	Casamino acids (10 mg.)	Methionine (1mg.)	Cysteine (0.01 mg.)	Cysteine (0.02 mg.)	Cysteine (0.1 mg.)	Cysteine (0.2 mg.)	Cysteine (0.5 mg.)	Cysteine (1 mg.)	Sodium thioglycolate (0.1 mg.)	Glutathione (0.1 mg.)	Ascorbic acid (0.1 mg.)	Hydroquinone (0.01 mg.)	Sodium hydrosulfite (0.1 mg.)
mg.	mg. units	mg. units	mg. units	mg. units	mg. units	mg. units	mg. units	mg. units	mg. units	mg. units	mg. units	mg. units	mg. units
0	2.2	0.55	0.13		0.4			0.4	0	0	0	0	0
1.0	4.6	3.2	2.0	3.4	5.2	4.6	1.1	0.4	3.6	2.1	2.1	0.9	0.9
2.0	5.0	4.0	2.1	4.4	10.0	5.6	1.1	0.6	7.8	3.5	2.3	1.4	5.2
5.0	8.2	9.2	6.2	6.2	>10	>10	2.7	1.1	10.0	9.0	7.5	4.0	10.0

*The extent of growth is expressed in terms of the apparent mg. units of yeast extract activity. The response to yeast extract alone in the pantothenic acid medium was used as standard.

Effects of Various Treatments on the Activity of Yeast Extract

Difco yeast extract was treated in the various ways indicated below, and the activities of the resulting preparations were determined.

Autoclaved at Natural pH--The activity of yeast extract was unaltered by autoclaving for 1 hour at the natural pH (5.8).

Autoclaved in N Acid--No change in activity was effected by autoclaving for 1 hour in N H_2SO_4.

Autoclaved in N Base--The activity of yeast extract was not changed by autoclaving for 1 hour in N NaOH.

Exposed to Diffuse Light--Exposure of solutions of yeast extract to diffuse daylight for 90 hours caused no reduction in activity.

Treated with Peroxide--Treatment with hydrogen peroxide caused an almost complete destruction of the activity of yeast extract. It was necessary to remove traces of unreacted peroxide by repeated evaporation of the sample to dryness because it was found that 30 γ of peroxide per tube would null the anti-cyanide activity of yeast extract at a 10 mg. level. The peroxide-treated sample had an apparent potency of 0.08.

Extracted with 70 Per Cent Alcohol--The undissolved portion of yeast extract obtained by refluxing the extract with 70 per cent ethyl alcohol had a potency of 0.16. The portion which was soluble in alcohol had a potency of 1.63.

Precipitated with Basic Lead Acetate--Yeast extract was precipitated with basic lead acetate (Horne's sugar reagent) following the procedure described by Snell and Strong.[98] After the removal of lead sulfide, the

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[98] E. E. Snell and F. M. Strong, Ind. Eng. Chem., Anal. Ed., 11, 346 (1939).

filtrate was aerated until it was free of hydrogen sulfide. Assay of this solution showed the contained solids to have a potency of 0.52. The presence of sodium acetate, which increases the amount of solids in the filtrate, appears to be responsible for this reduced potency. On an equivalent basis, there was no evidence of loss in activity.

Precipitated with Phosphotungstic Acid--Both the filtrate and the decomposed precipitate from the phosphotungstic acid precipitation of yeast extract were assayed. The reagent used to remove the excess phosphotungstic acid from the filtrate and to decompose the precipitate was barium hydroxide. The filtrate material had a potency of 0.93 and the precipitated fraction a potency of 4.2.

Adsorbed with Charcoal--Adsorptions were performed using Darco G-60; equal weights of the adsorbent and of yeast extract were used. The filtrates from adsorptions which were carried out at various pH levels (3 to 9) had essentially the same activity, the apparent potencies ranging from 0.24 to 0.32. Adsorptions were therefore carried out at the natural pH of yeast extract solutions. The fraction of yeast extract which was soluble in 70 per cent alcohol was also treated with charcoal, and the resulting filtrate material was found to have a potency of 0.35.

The adsorbed material was partially eluted from the charcoal cake by refluxing for 15 minutes with 20 volumes of an aqueous mixture of ammonium hydroxide (10 per cent) and ethyl alcohol (40 per cent). After filtration, the eluate was evaporated to dryness over a steam bath and the resulting preparation was assayed.

Further adsorption and elution of the eluate fractions was performed following the procedure used for the whole extract. Two treatments were

carried out on the first eluate from yeast extract, and the resulting preparations were designated as the second and third eluates. The eluate fraction from the alcohol-soluble yeast extract was retreated with charcoal, and the eluted material was also termed a second eluate. All of these eluate preparations were assayed both alone and combined with equivalent levels of the filtrate fraction from yeast extract. Dry weights were taken on aliquots of all of the eluate preparations, and these together with the assay results are shown in Table IX

TABLE IX

Activity of Eluate Preparations Alone and Combined with the Filtrate Material

Eluate preparations	Dry weight per mg. of original extract adsorbed	Apparent potency of eluate	Apparent potency* of eluate + filtrate
	mg.		
From yeast extract			
First	0.245	0.50	1.15
Second	0.135	0.37	0.86
Third	0.046	0	0.90
From alcohol-soluble yeast extract			
First	0.056	1.25	1.45
Second	0.037	0	1.50

*Filtrate and eluate preparations were added in equivalent amounts, e.g., an aliquot of the filtrate preparation derived from 1 mg. of yeast extract was added to that amount of eluate representing 1 mg. of yeast extract adsorbed, and the apparent potencies of such mixtures were determined. The potency of the filtrate preparation used was 0.35.

These data show that the activity of yeast extract depends on at least two factors which can be separated by means of charcoal adsorption.

A consideration of the assay values for the eluate preparations alone made it appear that a second and third adsorption entailed a complete loss of the active principle present in the first eluate, but assay values obtained in the presence of equivalent amounts of the filtrate material showed this not to be the case.

Effects of Certain Treatments on the Activity of Filtrate and Eluate Preparations

Peroxide Treatment--Since the anti-cyanide activity of yeast extract was almost completely destroyed by treatment with hydrogen peroxide, both the filtrate and eluate preparations were treated with this reagent in order to determine whether one or both of them was destroyed by this treatment. The peroxide-treated filtrate sample was assayed alone and in the presence of the eluate factor, and it was found to have essentially no activity. In order to be positive that the sample was not inhibitory, it was assayed in the presence of yeast extract (1 mg. per tube); values were the same as those obtained with the extract alone. In contrast to this, addition of the untreated filtrate preparation to yeast extract was found to produce a stimulation of growth greater than that obtained with the extract alone. Neither treated nor untreated filtrate samples had any effect on the amount of growth produced by casamino acids.

Peroxide treatment of the first eluates from the adsorption of yeast extract and the alcohol-soluble fraction of yeast extract caused little if any loss in activity. These samples were tested in the presence of yeast extract and found to have no inhibitory action. Neither the treated nor untreated eluate preparations had any effect on the stimulatory effect of

yeast extract on growth. On the other hand, when either of them was added to casamino acids, growth was enhanced more than it would have been if the effects of the eluate and casamino acids were merely additive.

Nitrous Acid Treatment--The filtrate and the first eluate from the adsorption of yeast extract were treated with nitrous acid (sodium nitrite + glacial acetic acid). After removal of the excess reagent by evaporation to dryness, the samples were tested alone and each in the presence of the other untreated preparation. Suitable controls were included in the test.

The results in Table X show that respective mixtures of equivalent amounts of the treated eluate-untreated filtrate and the untreated eluate-untreated filtrate have the same stimulatory effect on yeast growth. But the apparent potency of a mixture of equivalent amounts of the treated filtrate and the untreated eluate is approximately half as great as that of a similar mixture of the two untreated preparations. It appears, therefore, that the active material in the eluate is stable to treatment with nitrous acid but that the filtrate material is partially destroyed.

TABLE X

Effect of Nitrous Acid Treatment on the Activity of Filtrate and Eluate Preparations

Material tested	Apparent Potency
Filtrate	0.35
Filtrate, HNO_2 treated	0.16
Eluate	0.50
Eluate, HNO_2 treated	0.25
Filtrate + eluate	1.3*
Filtrate, HNO_2 treated + eluate	0.63*
Filtrate + eluate, HNO_2 treated	1.3*

*These values represent the potency of respective mixtures of equivalent amounts of the indicated filtrate and eluate preparations.

The foregoing results indicated definitely that adsorption with charcoal effected a separation of the substances in yeast extract which together are responsible for its action in reversing the cyanide inhibition of growth. The active material present in the unadsorbed fraction was designated as the filtrate principle and that in the adsorbed fraction which was eluted as the eluate principle. Further experiments were performed in an attempt to characterize and identify these two principles, most of the work being devoted to a study of the eluate principle.

FURTHER STUDIES ON THE FILTRATE PRINCIPLE

At this stage of the investigation, the following facts were known concerning the filtrate principle. It is soluble in 70 per cent alcohol, not adsorbed to any appreciable extent on Darco G-60, inactivated by treatment with hydrogen peroxide, and reduced in activity by treatment with nitrous acid. Since the activity of yeast extract was unaffected by autoclaving in acidic and basic media, it is probable that the filtrate principle is stable to these treatments.

The growth stimulation produced by (1) the filtrate preparation + the eluate preparation, (2) casamino acids + the eluate preparation, (3) the filtrate preparation + yeast extract, and (4) casamino acids + yeast extract was greater than could be accounted for on an additive basis. The similar behavior of the filtrate preparation and the casein hydrolysate suggested that possibly the filtrate principle is an amino acid. Therefore several amino acids were tested to see whether or not the filtrate principle was identical with one of them.

Activity of Various Amino Acids

The comparative effects of the filtrate principle and various amino acids were determined by growth studies in a cyanide medium supplemented with yeast extract (0.2 gm. per liter). The following compounds were added at levels from 200 to 800 γ per tube: valine, norleucine, isoleucine, leucine, glutamic acid, serine, threonine, proline, hydroxyproline, arginine, lysine, histidine, methionine, and α-amino-n-butyric acid. All of the compounds tested were inactive except norleucine which was inhibitory at the 200 γ level.

The possibilities remain that the filtrate principle is some amino acid other than the ones tested or that it is not one but a mixture of amino acids.

FURTHER STUDIES ON THE ELUATE PRINCIPLE

In order to study the eluate principle on a quantitative basis, it was necessary to modify the testing procedure which was used for the experiments involving yeast extract. In the first place, yeast extract could not be used as a standard since it contained both the filtrate and the eluate principles. The standard selected was an eluate preparation from yeast extract obtained by the application of the adsorption and elution procedures described previously; the growth-promoting activity of 1 mg. of this material was defined as 1 mg. unit. Again the term "potency" has been employed to indicate relative effectiveness of a material.

Ideally, a test medium contains an excess amount of all required substances except the one used as a standard. Therefore it was necessary to alter the culture medium when the standard material was changed. The cyanide medium, which previously had been modified by the substitution of pantothenic acid for β-alanine and the addition of cysteine, was supplemented with the filtrate principle. An amount of filtrate preparation equivalent to 1 gm. of yeast extract adsorbed was added per liter of medium. Though this amount did not supply an excess of the filtrate principle, it was used in order to avoid the higher blanks obtained with increased concentrations of the filtrate preparation. In testing natural materials, it was necessary to separate the eluate principle from any filtrate principle present. All other features of the testing procedure were identical with those previously described.

The growth response of F.B. yeast to various levels of the eluate standard is shown in Table XI.

TABLE XI

Growth Response of F.B. Yeast to the Eluate Principle

Amount of eluate preparation added	Galvanometer readings
mg.	
0	36
0.032	46
0.053	50
0.107	54
0.21	59
0.53	63

Activity of Various Known Compounds

A part of the study of the eluate principle was devoted to an attempt to identify the principle with some known substance. To this end, a large number of compounds were tested to see whether or not any one of them had a stimulatory effect on yeast growth comparable with that obtained with the eluate principle under the conditions employed. The activity of some of the compounds assayed had already been determined under other conditions.

Included among the compounds tested were amino acids, fatty acids, acid amides, hydroxyacids, purines, pyrimidines, steroids, diamines, terpenes, metal salts, the B vitamins, and representatives of other miscellaneous classes of compounds. None of the compounds was found to act as the eluate principle. For the most part, they were devoid of activity. Norleucine, however, was found to be inhibitory at a level of 50 γ per tube, while niacin, nicotinamide, and folic acid had a stimulatory effect.

The apparent potency of both niacin and nicotinamide at a 1 γ level was 7.4 and at a 20 γ level was 0.31. Similarly, for folic acid the potency at a 2.5 γ level was 3.0 but at a 25 γ level was only 0.52. Mixtures of niacin and folic acid and of nicotinamide and folic acid were prepared in which the concentrations of the components were varied, and these mixtures were tested. None of them behaved in the same manner as did the eluate principle. These substances may be responsible in part for the anti-cyanide action of the eluate principle in the presence of the filtrate principle, but they could not be shown to be entirely responsible.

Activity of Various Source Materials

Failure to identify the eluate principle with any known compound led to the investigation of various source materials. In order to determine the most suitable source for use in concentration procedures and at the same time to acquire some information concerning distribution of the principle, the following readily available materials were assayed: brewers' yeast extract, liver fraction B (Wilson's), liver fraction (Lederle's) malt extract, a fermentation residue (Curbay), dehydrated grass juice (Cerophyl Laboratories), an enzymatic digest of casein (amigen), rice bran concentrate (vitab), beef muscle, beef liver, and fresh spinach. The eluate principle was separated from any filtrate principle present in these materials by resorting to the same adsorption and elution procedure as that used on yeast extract. For those substances which were slightly soluble in water, suspensions of the finely divided material were steamed for 30 minutes, filtered, and the resulting extract was adsorbed with Darco G-60 and subsequently eluted.

Assay results for these various eluate preparations are shown in Table XII.

TABLE XII

Activity of Eluates of Various Source Materials

Source material	Apparent potency of eluate
Difco yeast extract	1.0 (Standard)
Brewers' yeast extract	0.50
Liver fraction B	0.43
Liver fraction (Lederle's)	0.14
Malt extract	0.12
Fermentation residue	0.13
Grass juice	0.23
Amigen	0.06
Vitab	0.13
Beef muscle	0.73
Beef liver	0.55
Spinach	0.25

The eluate principle was found to be present in all of the materials which were assayed, though none of the substances tested contained as much of the principle as does Difco yeast extract. Therefore, the use of Difco yeast extract as the source material for the eluate principle was continued.

Properties of the Eluate Principle

The following information concerning the properties of the eluate principle had been accumulated thus far. The principle is adsorbed on charcoal from which it can be eluted by an aqueous mixture of alcohol and ammonia. It must be soluble in 70 per cent alcohol since the eluate obtained from the alcohol-soluble fraction of yeast extract contained the active principle. Neither peroxide treatment nor treatment with nitrous

acid appeared to alter the activity of the principle. When the principle is added to the filtrate resulting from the charcoal adsorption of yeast extract or to casamino acids, the observed growth stimulation represents a more than additive effect. By inference, it appeared that the active material in eluate preparations was stable to acid and base treatments since the activity of yeast extract was unaltered by autoclaving in N acid and in N base.

Eluates were prepared from yeast extract samples which had been autoclaved for 1 hour in N H_2SO_4 and N NaOH, respectively, and assay of these preparations showed that the principle had undergone no change in activity.

A solution of the standard eluate preparation (0.1 per cent) which was exposed to ultraviolet light for 48 hours showed no loss in activity.

The eluate principle was found to be precipitated by phosphotungstic acid treatment and partially precipitated by treatment with either basic lead acetate or ammoniacal silver nitrate.

Some concentration of the eluate principle was effected by treating yeast extract with 95 per cent ethyl alcohol under reflux. The eluate prepared from the soluble fraction was found to be approximately 6 times as active as the standard preparation. Apparently some of the inert material which is adsorbed and eluted along with the eluate principle is insoluble in 95 per cent alcohol.

The eluate principle was subjected to electrolysis at high voltage in the electrical transport apparatus described by Williams and Truesdail.[99] The active principle was found to be distributed throughout the four-cell system employed; therefore it appears that the activity of eluate preparations does not depend on a single substance.

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[99] R. J. Williams and J. H. Truesdail, J. Am. Chem. Soc., 53, 4171 (1931).

Exposure of solutions of the standard eluate (0.1 per cent) to direct sunlight for 18 hours caused little if any loss in activity. It was observed, however, that dilute solutions of the eluate principle which were kept in the refrigerator for a month's time underwent deterioration in activity (approximately 50 per cent).

An attempt to concentrate the eluate principle by repeated adsorption and elution was not very successful. The standard eluate was subjected to six consecutive treatments, and the activity of the resulting preparation was only approximately 8 times that of the standard. Moreover, about 50 per cent of the active material was lost during the process.

SUMMARY

Yeast extract and other biological materials have been shown to reverse the inhibition of yeast growth produced by cyanide. No known compound or mixture of compounds was found which could duplicate the anti-cyanide action of yeast extract, although methionine and cysteine were found to have some anti-cyanide activity. Excess pantothenic acid had a beneficial effect on growth under certain conditions.

It was demonstrated that the action of yeast extract depended on at least two substances which could be separated by charcoal adsorption. Though these two substances had certain properties in common, they had other dissimilar properties which indicated that they were not identical. Attempts to identify these active principles were not successful. The potency of the eluate principle was increased approximately 8 times by repeated adsorption and elution from charcoal. Indirect evidence indicates that the activity of both filtrate and eluate principles is due to more than a single substance.

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